Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 69 DECEMBER, 1948 No. 3

SECTION MEETINGS

| | |
|---|---|
| ILLINOIS | |
| University of Chicago | October 26, 1948 |
| MINNESOTA | |
| University of Minnesota | November 17, 1948 |
| SOUTHERN CALIFORNIA | |
| California Institute of Technology | November 3, 1948 |
| SOUTHWESTERN | |
| Randolph Field, Texas | November 5, 1948 |
| WISCONSIN | |
| Marquette University | November 5, 1948 |

16732

## Relaxin in the Ovary of the Domestic Sow *(Sus scrofa* L.).*

F. L. HISAW AND M. X. ZARROW.
*From the Biological Laboratories, Harvard University, Cambridge, Mass.*

The recent work of Albert, Money and Zarrow[1] indicates that a much higher concentration of relaxin is found in the whole, fresh ovary of the sow than in the defatted, dried corpora lutea.[2-4] They[1] also found that the hormone is distributed throughout the ovary with the exception of the follicular fluid, and that during pregnancy its concentration is 500 to 1500 times greater than during a normal estrous cycle. The present investigation was undertaken for the purpose of determining quantitatively the relaxin content of the ovary throughout pregnancy and also to find when relaxin first appears in the ovary both during pregnancy and the normal estrous cycle. In addition, studies were made of the blood, uterus and placenta for relaxin content.

*Experimental procedure and methods.* In order to avoid the possible loss of relaxin as a consequence of the number of manipulations in the extraction of the tissues, purification was carried out only to the point of furnishing a preparation suitable for assay purposes. Essentially the technic used was that described as step No. 1 by Albert, Money and Zarrow.[1] The tissues were obtained on the slaughtering floor and at the same time data were secured as to the age of the sow, condition of the ovary (follicles or corpora

* Aided by a grant from the United States Public Health Service to Professor Frederick L. Hisaw.

1 Albert, A., Money, W. L., and Zarrow, M. X., *Endocrinol.*, 1947, **40**, 370.

2 Fevold, H. L., Hisaw, F. L., and Meyer, R. K., *J. Am. Chem. Soc.*, 1930, **52**, 3340.

3 Abramowitz, A. A., Money, W. L., Zarrow, M. X., Talmage, R. V. N., Kleinholz, L. H., and Hisaw, F. L., *Endocrinol.*, 1944, **34**, 103.

4 Albert, A., Money, W. L., and Zarrow, M. X., *Endocrinol.*, 1946, **39**, 270.

lutea) and, if pregnant, the length of the fetus. The tissues were brought immediately to the laboratory and the preparation of the extracts for assay was started.

The tissues were first ground and extracted in the cold with 6 volumes of 3% HCl for 48 hours. At the end of the first extraction period the mixture was made up to 4% NaCl and the supernatant fluid separated from the residue by centrifugation. The residue was then re-extracted in the same manner for an additional 24 hours and discarded. The two supernatant fluids were combined, adjusted to pH 7.0 and dialyzed against running tap water. Adequate dilutions were prepared and the sample assayed in castrated guinea pigs by means of the relaxation of the symphysis pubis. In a few instances when the extract was too dilute, the preparation was concentrated by adding 5 volumes of cold acetone and dissolving the precipitate in a suitable volume of water.

In the present assay procedure the guinea pigs were injected with 1 $\mu$g of estradiol daily for 3 days instead of the usual 4 days and the material to be tested was injected on the morning of the fourth day. Six hours later the pelves of the animals were palpated according to the technic of Abramowitz, *et al.*[4] A guinea pig unit (G.P.U.) was defined as that amount of relaxin which produced relaxation of the symphysis pubis in two-thirds of a group of 12 or more guinea pigs. The assay was carried out by preparing a series of dilutions and determining the concentration of relaxin which gave a unit response.

It is important to note that several precautions were carefully observed. Only animals in good nutritional state and weighing 400 to 800 g were used. Furthermore, all guinea pigs were palpated prior to the start of the estradiol treatment and also just prior to the injection of the sample of relaxin. In both instances all guinea pigs with relaxed or questionably relaxed pelves were discarded. Finally, it is important to mention that it is possible to distinguish gradations in the response of the guinea pigs. However such distinctions may lead to considerable error as the test is in a large measure based on subjective judgment. Consequently, only an unquestionable relaxation of the symphysis was accepted as a positive response while doubtful or non-relaxed symphyses were considered negative.

*Results.* Ovaries were obtained from 15 pregnant sows with fetuses ranging in length from 3/8 to 10 3/8 inches and assayed for relaxin content on a total of 510 castrated guinea pigs. Prior to carrying out these assays, an extract was prepared of a single ovary from a sow in early pregnancy and a dose-response curve obtained. It may be seen in Fig. 1 that the results give a typical sigmoid curve similar in shape to the curve obtained with extracts of dried corpora lutea,[3] thus indicating that the assay reported pre-

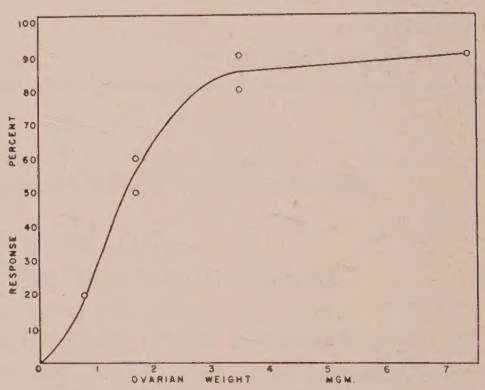



FIG. 1.

The dose-response curve of relaxin obtained from the ovary of a pregnant sow. Mg equivalent of fresh ovary are plotted against the percentage of guinea pigs showing pubic relaxation.

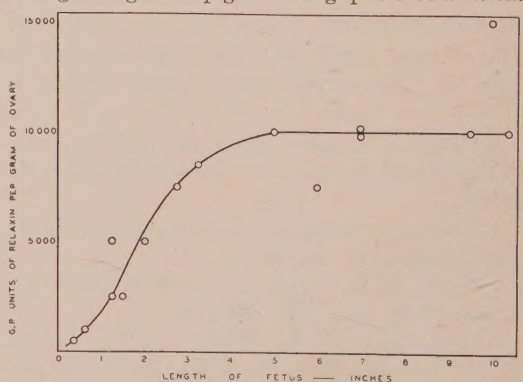



FIG. 2.

The relaxin content of the ovary of the pregnant sow plotted against the average length of the fetuses as an indication of the stage of pregnancy.

TABLE I.
Concentration of Relaxin in Blood, Placenta, Ovary, and Other Tissues of the Sow.

| Age of pig | Tissue | Stage of cycle or length of fetus | No. of tests | Relaxin content G.P.U. |
|---|---|---|---|---|
| Gilt | Ovary | Immature | 4 | <1.0 per g |
| Mature | " | Follicular | 2 | <1.0 " " |
| " | " | Luteal | 3 | 2.5 to 5 " " |
| " | Follicular fluid | Follicular | 2 | <1.0 " 2 ml |
| " | Placenta | 4½-inch fetus | 1 | 0.5 " g |
| " | " | 6 " " | 2 | 2.5 " " |
| " | " | 10 " " | 2 | 0.5 " " |
| " | Blood | 5½ " " | 1 | 2.0 " 1 ml |
| " | " | 7 " " | 2 | 2.0 " " " |
| " | Uterus | Pregnant | 1 | <1.0 " g |
| " | Thymus | — | 1 | <0.1 " " |
| " | Thyroid | — | 1 | <0.1 " " |
| " | Liver | — | 1 | <1.0 " " |
| " | " | — | 1 | <0.1 " " |

viously is applicable to the present material.

Assays of the ovaries of pregnant sows (Fig. 2) indicate that the relaxin concentration rapidly increases during early pregnancy, and reaches a plateau at approximately 10,000 G.P.U. per gram of fresh ovary when the fetus is about 5 inches in length. The earliest stage of pregnancy studied was one in which the fetuses were ⅜ of an inch in length. The ovaries of this sow contained 500 G.P.U. of relaxin per gram of fresh tissue. Therefore, the relaxin content of the ovary increased some 20-fold between this early stage and that at which the fetuses attained a length of 5 inches.

An extract of the ovaries of a sow with fetuses 9½ inches long was used for making a comparison of the guinea pig and mouse assay procedures. The guinea pig assay was carried out as previously described. In the mouse assay, groups of 15 castrated female mice previously primed with estradiol were used and a unit defined as that amount of relaxin which would produce relaxation in the symphyses of two-thirds of the animals. There was complete agreement between the results of the two methods of assay as both showed a concentration of 10,000 G.P.U. of relaxin per gram of fresh ovary.

In view of the extremely high concentrations of relaxin in the ovaries of the pregnant sow it was felt desirable, for the purpose of comparison, to examine the ovaries of the gilt and of the sow during a normal estrous cycle. The tissues were collected and assayed by the guinea pig method in the same manner as described above. These assays gave negative results, at a level corresponding to 1 G.P.U. of relaxin per gram of fresh tissue, for both the ovary of the gilt and that of the sow in the follicular phase of the estrous cycle. Examination of the follicular fluid also gave negative results. However, during the luteal phase, the ovary was found to contain 2.5 to 5 G.P.U. per gram.

The relaxin content of the blood, placenta and other tissues of the pregnant sow also was determined (Table I). Several assays were carried out on the blood serum of 2 pregnant sows, one of which had fetuses of 5½ inches and the other fetuses of 7 inches. In both cases the blood was found to contain 2 G.P.U. per ml. The placentas of 3 animals having fetuses 4½, 6, and 10 inches in length were found to contain 0.5 to 2.5 G.P.U. per gram and no correlation with length of gestation was observed. Assays for relaxin in the uterus, thyroid, thymus and liver gave negative results.

*Discussion.* These observations indicate that relaxin is primarily a hormone of pregnancy. It is found in the ovary in small amounts (2.5 to 5.0 G.P.U. per g) during the estrous cycle, associated with the presence of a functional corpus luteum, but it begins to increase very early in pregnancy

and attains a maximal concentration of approximately 10,000 G.P.U. per g of ovarian tissue by the time the fetuses are 5 or 6 inches in length. It is rather surprising, in view of this high concentration in the ovaries, to find only 2 G.P.U. of the hormone per ml of blood serum in the pregnant animal. This is only one-fifth of the concentration found in the blood serum of pregnant rabbits.[5] However, it is 4 times the concentration found in pregnant guinea pigs[6] which indicates a wide species variation.

While the ovary must be regarded as the major source of relaxin in the pregnant sow, small amounts (0.5 to 2.5 G.P.U. per g) were also found in the placenta. Compared to the concentrations obtained in the ovary this amount is extremely small; nevertheless it is significant to note that in both the guinea pig[6] and the rabbit,[7] relaxin has been found in the placenta.

5 Marder, S. N., and Money, W. L., *Endocrinol.*, 1944, **34**, 115.

6 Zarrow, M. X., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 488.

7 Zarrow, M. X., Unpublished observations.

The lack of relaxin in the ovary of the gilt and in the follicular fluid and ovary of the sow in pre-oestrus indicates that the corpora lutea are of importance in the formation of relaxative substance. The results of Albert, Money and Zarrow[1] show that relaxin is present in both the corpora lutea and the non-luteal tissue of the ovary of a pregnant sow. This may indicate that relaxin is formed in the luteal tissues and diffuses out to the surrounding area or that there is an extra-luteal site which forms relaxin under the stimulation of a corpus luteum.

*Summary.* No relaxin is found in the ovaries of the gilt nor of the sow during the follicular phase of the estrous cycle. There is, however, 2.5 to 5 G.P.U. of relaxin per gram of ovarian tissue present during the luteal phase of the cycle. The amount of relaxin in the ovaries increases rapidly during pregnancy and reaches a maximal concentration of approximately 10,000 G.P.U. per gram of tissue by the time the fetuses attain a length of 5 or 6 inches. The blood at mid-pregnancy contains 2 G.P.U. per ml while the placenta has 0.5 to 2.5 G.P.U. per gram.

16733

## Electrophoretic Changes in Plasma Proteins in Patients with Pneumococcic Infections.

ROBERT A. BRUCE AND ERIC L. ALLING. (Introduced by W. S. McCann.)
(with the technical assistance of Anne D. Barnett.)

*From the Chest Laboratory of the Department of Medicine and the Departments of Radiology and Medicine, University of Rochester School of Medicine and Dentistry, and the Medical Clinic of the Strong Memorial and Rochester Municipal Hospitals, Rochester, N. Y.*

Alterations of the plasma proteins in relation to successive stages of pneumococcic infections (either pneumonia or meningitis), and to important antecedent factors influencing protein metabolism, are reported in this paper. Blix,[1] who studied the plasma of pneumonia patients electrophoretically, found that the alpha-globulin concentration was more than twice normal, and gamma globulin was reduced from 76 to 64% of the total globulin concentration. Longsworth[2] confirmed these findings concerning alpha-globulin, and subsequent observers have shown that alpha-globulin generally increases in febrile reactions associated with tissue destruction, such as infectious diseases, coronary thrombosis, frac-

1 Blix, G., *Z. f. die gesamte Exp. Med.*, 1939, **105**, 595.

2 Longsworth, L. G., Shedlovsky, T., and MacInnes, D. A., *J. Exp. Med.*, 1939, **70**, 399.

TABLE I.
Selection of Patients with Pneumococcic Infections.

| Age, yrs | Pre-existing complications | Days of illness on which observations were made | Additional protein therapy | Recovered |
|---|---|---|---|---|
| | Primary Meningitis. | | | |
| 32 | None | 1,3 | Serum | Yes |
| 38 | Pituitary adenoma | 1,4 | " | " |
| 50 | Skull fracture | 1,6 | " | " |
| 60 | Cirrhosis | 4,7 | " | No |
| | Primary Pneumonia | | | |
| 46 | None | 18,25,79 | | Yes |
| 28 | " | 4,6,14,33 | Gamma globulin | " |
| 34 | " | 2,4 | " " | " |
| 39 | Alcoholism | 21,36 | Serum | " |
| 57 | " | 21,24,49 | " | " |
| 46 | " chr. osteomyelitis | 4 | | No |
| 49 | " | 19 | | Yes |
| 70 | " | 10,14,31,46 | Gamma globulin | " |
| 62 | " bronchiectasis | 11,15,29 | " " | " |
| 42 | " | 8,9 | " " | " |
| 43 | " | 7,8,17 | " " | " |
| 79 | Congestive failure | 8,17,24 | | No |
| 70 | Emphysema | 14,28 | | Yes |
| 71 | Heart disease | 3,21 | | " |
| 74 | Congestive failure | 5,6,32 | " " | " |
| 78 | Heart disease | 36 | | " |
| 69 | Diverticulitis | 6 | | " |
| 74 | Cachexia senilis | 4 | | " |
| 65 | Multiple myeloma | 1,5 (months) | | " |

tures and burns.[3] Leutscher[4] reported greatly decreased electrophoretic albumin and increased fibrinogen concentrations in lobar pneumonia, as well as lesser increments in beta and gamma globulins. Pleural fluids similarly fractionated were found to contain the same constituents as serum, but in lower concentrations.

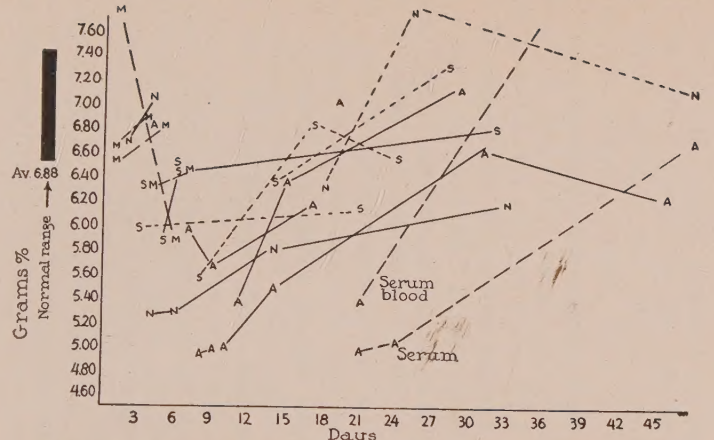
*Selection of patients.* During 1946, 23 patients with pneumococcic infections were selected from the Medical Clinic of the Rochester Municipal and Strong Memorial Hospitals for this study. As indicated in Table I, 4 of these patients had primary meningitis, and 19 pneumonia. In addition to routine treatment with sulfadiazine and/or penicillin, 6 were also treated with type specific rabbit antiserum because of the severity of the infection, and 7 others were given supplemental intramuscular injections of immune human gamma globulin* to investigate its effects.

*Methods.* The venous blood samples were drawn in dry oxalate flasks under fasting conditions to minimize lipemia, and insofar as possible with only momentary stasis. All but 3 plasma samples had a specific gravity of 1.022 to 1.026 (copper sulphate method); one from a patient with pneumonia and hepatic cirrhosis was too low to read on one day and 1.021 when repeated the following day, and 2 others were 1.029 from acutely ill dehydrated patients. Electrophoresis was carried out in the tall form of the 11 ml Tiselius cell, in diethyl-barbituric acid buffer of pH 8.5 and ionic strength of 0.1. Plasma was diluted with an equal volume of buffer and dialyzed through cellophane against 2 liters of similar buffer. Current was passed for 3 hours with a field strength of 6.8 volts per

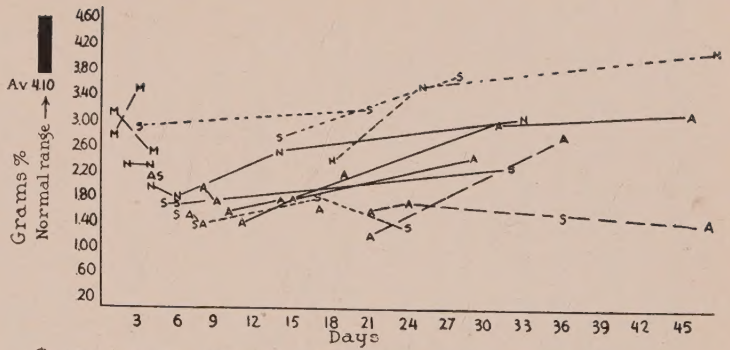
[3] Stern, K., and Reiner, M., *Yale J. Biol. and Med.*, 1946, **19**, 67.

[4] Leutscher, J. A., *J. Clin. Invest.*, 1941, **20**, 99.

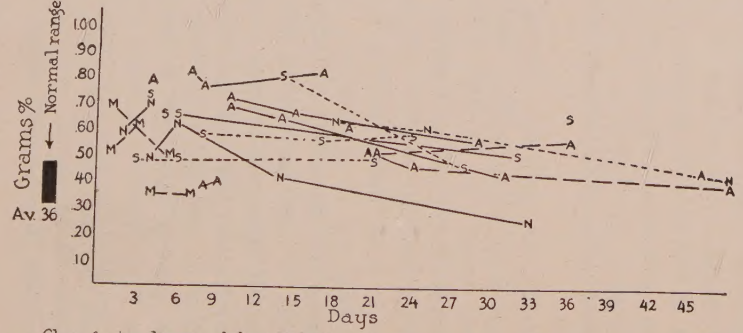
* The immune human gamma globulin was furnished by Sharpe and Dohme, Philadelphia, through the kindness of Dr. Charles A. Janeway, Boston. 50 ml of this material provided the antibody equivalent of 1200 ml of pooled normal human plasma, or not quite one-half of the estimated circulating gamma globulin in the blood.



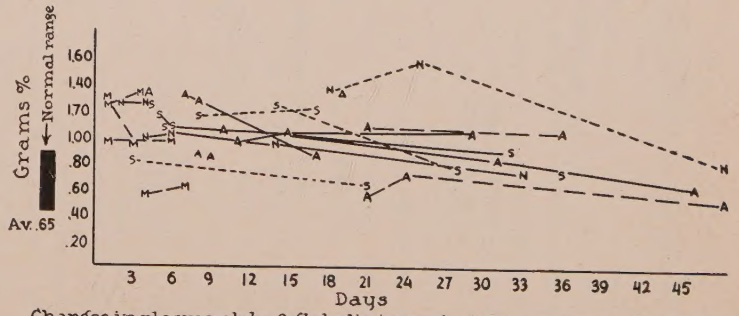

Changes in plasma total protein concentration in pneumococcic infections




Changes in plasma albumin concentration in pneumococcic infections

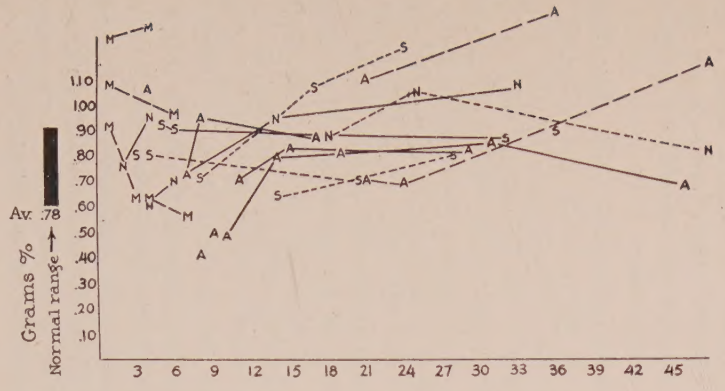



Changes in plasma alpha 1 globulin concentration in pneumococcic infections

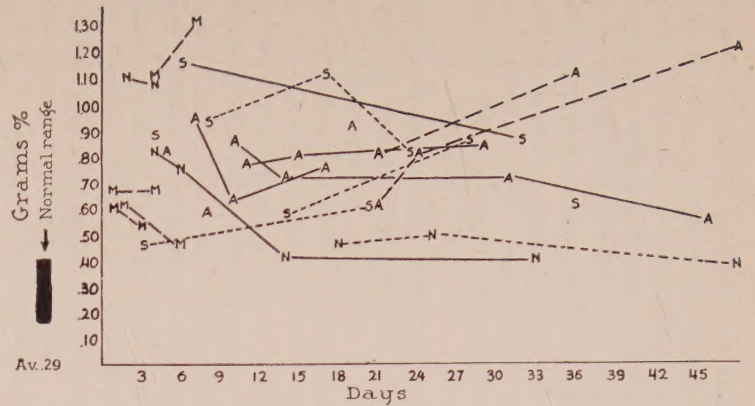



Changes in plasma alpha 2 globulin concentration in pneumococcic infections

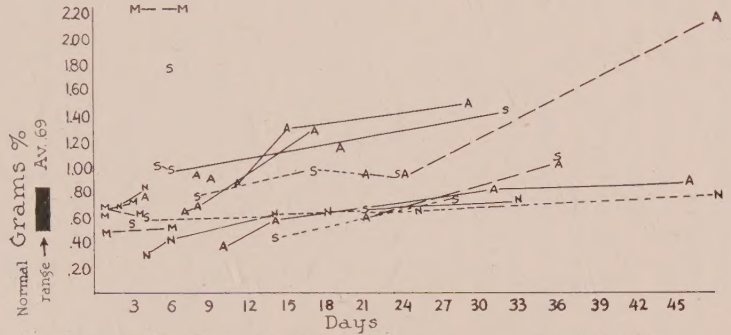
FIG. 1. Serial Changes in Plasma Protein Fractions in Pneumococcic Infections.



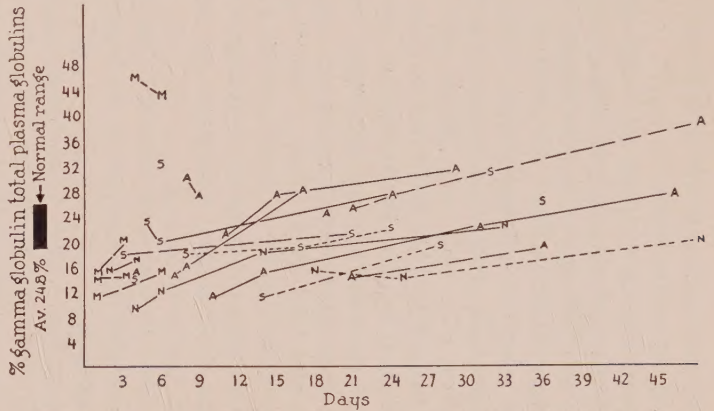

Changes in plasma beta globulin concentration in pneumococcic infections




Changes in plasma fibrinogen concentration in pneumococcic infections




Changes in plasma gamma globulin concentration in pneumococcic infections




Changes in % gamma globulin concentration in combined globulin fractions in pneumococcic infections

cm. Further details of this technic may be found in a previous paper.[5]

*Results.* The alterations in plasma proteins determined electrophoretically in relation to the duration of the pneumococcic infection are shown in Fig 1. In addition, the symbols indicate patient-groups: primary meningitis (M), senile (S), history of chronic alcoholism (A), and those whose previous health was normal (N). Differences in therapy are indicated by the connecting lines: small broken lines indicate routine treatment; large broken lines indicate supplemental use of antiserum; and solid lines indicate those treated with gamma globulin. For comparative purposes the range and mean values for the same fractions in 17 normal individuals are indicated in each graph.

All *total protein* levels obtained initially were somewhat depressed, except for 1 patient with meningitis and dehydration (plasma specific gravity 1.029). The blood samples from the meningitis group were obtained early in the infection and exhibited an average level of 7.07 grams%, whereas those with pneumonia were obtained later in the first and second weeks of the illness and had an average level of 5.88 grams%. There was considerable scatter, with a tendency to reach the lowest levels in the second week, and then to increase during convalescence. This parallels the observations of Rutstein, *et al.*, on plasma volume alterations in pneumonia.[6] The pneumonia patients with an alcoholic background had an average total protein concentration of 5.55 g%, whereas the non-alcoholic group averaged 6.07 g%. These values are based on samples taken from 2 to 36 days in the course of the illness, and hence are not strictly comparable. The lowest total protein of 4.9 g% was obtained from an alcoholic patient however.

The plasma *albumin* levels were low in all the initial blood samples studied. Hypoalbuminemia progressed until the second week of the infection, and all but 2 patients continued to exhibit a subnormal albumin concentration up to a month after the onset of the infection. The lowest level for non-alcoholic patients was 1.76 g%, whereas the elderly and alcoholic groups exhibited a more marked hypoalbuminemia ranging from 1.35 to 1.74 g% during the first 2 weeks. This is in contrast to the normal range of 3.7 to 4.6 g% by this method.

The *alpha-globulins* were increased up to twice normal concentrations during the first week. The 2 exceptions to this trend were patients with advanced cirrhosis, one of whom died of hepatic insufficiency. The increased alpha-globulin levels gradually declined to normal in over a month. The *beta globulin* levels varied considerably; the highest initial level occurred in a patient with pituitary adenoma who had an intracranial-paranasal fistula complicated by meningitis; and the lowest levels were in 2 patients with chronic alcoholism. During convalescence the beta globulin showed marked increases in 3 patients, one of whom was an elderly man with a history of congestive heart failure who suffered 3 relapses of pneumonia before a fatal outcome; and the other 2 were chronic alcoholics with pneumonia treated with antipneumococcal rabbit serum.

The *"fibrinogen fraction"* was considerably increased in all patients, and returned to normal during convalescence in only 2 patients. These 2 had pneumonia without any antecedent complications. The highest value of 1.3 g% was obtained just prior to death in the patient with meningitis and hepatic insufficiency. Two others, who showed progressive hyperfibrinogenemia, had chronic alcoholism, were severely ill with pneumonia, and showed jaundice. It should be noted that the fibrinogen values obtained by electrophoresis contained unknown amounts of gamma-1 globulin, so that the increases observed cannot be considered pure fibrinogen. Hence we have used the term "fibrinogen fraction."

*Gamma globulin* concentrations of greater than 0.9 g% were seen in the acute phase of the illness or in convalescence in 10 patients. Of these, 4 had chronic alcoholism; 3 had

[5] Zeldis, L. J., and Alling, E. L., *J. Exp. Med.*, 1945, **81**, 515.

[6] Rutstein, D. D., Thomson, K. J., Tolmack, D. M., Walker, W. H., and Floody, R. J., *J. Clin. Invest.*, 1945, **24**, 11.

hepatic cirrhosis; 2 had heart failure with hepatic congestion; and 1 had multiple myelomata. Thus the abnormal increments in the gamma globulin levels appeared to be related to underlying disease of the liver or bone marrow. In the electrophoretic fractionation of the plasma proteins in the myeloma blood, there was a protein having the mobility of gamma globulin with a concentration of 5.9 g% as determined by the area under the peak. When this patient's plasma was studied again 5 months later, there was no change in this peak. Only 2 patients had subnormal gamma globulin levels. Each of these gave a history of previous pneumonic infections several years before. Both were given 50 ml of immune human gamma globulin intramuscularly in addition to the routine treatment. Subsequent electrophoretic patterns of their blood showed increments in the gamma globulin fraction. Four out of 5 other patients similarly treated with intramuscular gamma globulin showed appreciable increases in the plasma concentrations of this fraction following the injection (Fig. 1). The amount of gamma globulin injected in these 7 patients represented about 7.0 g, and the maximum average increase in concentration was 0.45 g%, or about 65% of the average normal level. Absorption was delayed as there was no prompt initial rise. Since, however, 4 of the 7 so treated were known to have had chronic alcoholism and another had had decompensated heart disease, it is difficult to attribute the greater increments in this fraction to the material administered, although it is possible that the restoration of normal levels was significant in the only 2 patients with abnormally low initial gamma globulin levels. During convalescence there was a relative increase in the gamma globulin concentration in all the patients studied, but this was attributed in part to the concomitant decline in alpha-globulins.

*Discussion.* Pneumococcic infections, in common with other acute infectious diseases and severe bodily trauma,[3] initiate non-specific alterations in the protein metabolism. The present findings of hypoalbuminemia, increased alpha-globulins and fibrinogen are in accord with the findings of previous observers.[1,2] When evaluated chronologically, however, from the onset of the infection, it is apparent that the alpha-globulins and fibrinogen increase promptly and decline gradually in convalescence; whereas the hypoalbuminemia becomes progressively more severe, reaching the lowest levels in the second week of the infection and slowly is restored to normal over the following month. The intensity of the hypoalbuminemia, and in turn the hypoproteinemia, is considerably influenced by antecedent factors adversely affecting liver function, such as malnutrition, chronic alcoholism, cirrhosis and chronic passive congestion. Furthermore the severity of the electrophoretic hypoalbuminemia is greater than is recognized by chemical determinations. Dole[7] has shown that the Howe separation[8] is faulty, due largely to the solubility of much of the alpha-globulins in the concentration of sodium sulfate employed in the Howe technic. Hence the hypoalbuminemia tends to be obscured by the concomitant increase in alpha-globulins during acute febrile illnesses which are incompletely salted out by the clinical method of separation. The hypoalbuminemia in pneumonia may be the result of poor dietary intake of nitrogen, nitrogen catabolism, losses of protein in exudate and urine, and possibly impaired synthesis of protein. In experimental dogs, poor dietary protein intake and plasmaphoresis have been shown to diminish the albumin level and increase the alpha-globulins.[5]

Previous variations in the reported findings of gamma globulin concentrations in pneumonia[1,4] may have been due to the selection of patients and the relation to the onset of infection. Our findings indicate abnormally low levels early in the infection in occasional patients, a relative increase in concentration during convalescence, and an abnormally high level in those patients having impaired liver function prior to the onset of pneumococcic infection. The supplemental intramuscular

7 Dole, V. P., and Braun, E., *J. Clin. Invest.*, 1944, **23**, 708.

8 Howe, R. E., *J. Biol. Chem.*, 1921, **49**, 109.

injections of gamma globulin† may have been responsible for the increases in plasma levels which occurred in the 2 patients with low values.

*Summary.* Multiple electrophoretic determinations of the plasma proteins of 23 patients with pneumococcic pneumonia or meningitis have been made in relation to the duration of the infection and the importance of antecedent factors. Hypoproteinemia, due largely to significant progressive hypoalbuminemia, together with early increases in alpha-globulins and fibrinogen have been observed. Malnutrition, chronic alcoholism, cirrhosis and chronic passive congestion of the liver were found associated with more severe degrees of hypoalbuminemia. Diminished plasma albumin levels were seldom restored to normal within a month from the onset of the infection. The effects of intramuscular injections of immune human gamma globulin were investigated, with inconclusive results.

† Dr. W. Addison Clay assayed the protective power of gamma globulin in type 27 pneumococcic peritonitis in 40 mice and found a reduction of gross mortality from 95 to 55%. By serial dilutions the protective power was estimated to be of the order of one ten thousandth of that of immune rabbit serum.

16734

## Effects of Albumin on Hypoalbuminemia in Pneumococcic Pneumonia.

ROBERT A. BRUCE* AND ERIC L. ALLING. (Introduced by W. S. McCann.)
(with the technical assistance of Anne D. Barnett.)

*From the Chest Laboratory of the Department of Medicine, and the Department of Radiology and Medicine, University of Rochester School of Medicine and Dentistry, and the Medical Clinic of the Strong Memorial and Rochester Municipal Hospitals, Rochester, N.Y.*

A previous report[1] confirmed the findings of others that patients with pneumococcic infection exhibit hypoalbuminemia within the first 2 weeks of the infection. Results obtained by electrophoresis of plasma proteins showed depression of albumin concentrations to as low as 1.4 to 1.8 g%, and that normal concentration was not often established within a month after the infection. Such marked hypoalbuminemia was considered to be the result of protein loss in exudate and urine in addition to protein break-down and malnutrition during the acute phase of the illness. Because of this hypoalbuminemia the effects of intravenous albumin therapy were investigated. This report presents the metabolic changes as well as some of the secondary effects of such therapy on cardio-respiratory functions.

*Patients and procedures.* During 1947 eight patients with severe pneumococcic lobar pneumonia were selected, and in addition to the routine chemotherapy of the infection 2 were controls, and 6 were treated with albumin. Observations on still another patient are included because of a fatal outcome following albumin therapy. The 6 albumin-treated patients and one control were placed on a special liquid diet† of known nitrogen content a day or two after the restoration of normal temperature following the febrile reaction. Twenty-four hour urinary collections were made to determine urinary nitrogen ex-

* Bertha Hochstetter Buswell Research Fellow in Medicine.

1 Bruce, R. A., Alling, E. L., and Barnett, A. D., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 398.

† The diet consisted of 1200 ml of powdered whole milk, eggs, yeast, and sugar in addition to orange juice. There was 17.7 g of nitrogen in this mixture. To satisfy some patients, the amount of diet had to be increased to 2400 ml volume.

TABLE I.
Results of Albumin Administration in Patients with Pneumococcic Lobar Pneumonia.
A. Effects on Plasma Albumin Levels and Protein Metabolism.

| Patient | Day of illness when alb. was started | Plasma albumin conc., g/100 ml | | | Protein intake, g | | Apparent retention in protein during period of observation, g |
|---|---|---|---|---|---|---|---|
| | | Before | After | 1 wk later | Dietary | Alb. i.v. | |
| J.S. | Control | 2.65 | 2.85 | 2.88 | 542 | — | 62 |
| C.M. | " | 3.20 | | 3.24 | * | — | — |
| D.P. | 5 | 2.48 | 3.46 | 3.48 | 611 | 100 | 245 |
| W.D. | 10 | 2.68 | 3.74 | 3.91 | 743 | 100 | — |
| A.M. | 7 | 2.51 | 3.64 | 3.78 | 743 | 100 | 244 |
| S.W. | 11 | 1.58 | 3.19 | 3.23 | 472 | 125 | 151 |
| E.E. | 9 | 1.25 | 2.26 | — | 890 | 125 | 598 |
| C.S. | 7 | 2.54 | 4.01 | 3.32 | 986 | 170 | 363 |

*This patient was permitted an unrestricted diet, hence the nitrogen intake could not be determined.

B. Effects on Plasma Globulin Levels.

| Patient | Alpha-1 | | Alpha-2 | | Beta | | Fibrinogen | | Gamma | |
|---|---|---|---|---|---|---|---|---|---|---|
| | b* | a | b | a | b | a | b | a | b | a |
| J.S. | .66 | .52 | 1.33 | 1.19 | .79 | .95 | .95 | .89 | .58 | .75 |
| C.M | .58 | .58 | 1.10 | 1.24 | .87 | .92 | .97 | .88 | .60 | .96 |
| D.P. | .69 | .51 | 1.29 | .92 | .92 | .75 | .72 | .45 | .80 | .58 |
| W.D. | .67 | .46 | 1.21 | .91 | 1.02 | .95 | .67 | .58 | .99 | .74 |
| A.M. | .64 | .47 | .98 | .76 | .73 | .82 | .71 | .50 | .60 | .82 |
| S.W. | .60 | .47 | .99 | .86 | .91 | .93 | .82 | .92 | 1.01 | 1.14 |
| E.E. | .65 | .58 | .95 | .88 | .82 | .71 | .93 | 1.23 | 1.06 | 1.18 |
| C.S. | .48 | .35 | .95 | .70 | 1.09 | .87 | .93 | .50 | .70 | .68 |

* "b" represents the concentration before the albumin was administered, and "a" represents the concentration after the albumin.

cretion, whereas the fecal nitrogen excretion was estimated to be 1.0 g per diem. Such a simplified nitrogen balance study was maintained for 5 to 7 days during which time the treated patients received 25 g (100 ml) of salt-poor human albumin‡ intravenously daily for 4 to 6 days. Venous blood samples for plasma electrophoresis were obtained the morning before, the morning after, and again a week after the course of albumin. The technic of electrophoresis was the same as previously described.[1,2] Electrophoresis of the plasma was done on the second control patient who was permitted an unrestricted hospital diet *ad libitum*. Serial determinations of pulse rate, blood pressure, venous pressure, respiratory rate, breath-holding time, cardiac output (ballistocardiographic method§), and pulmonary capacities including total and vital capacity as well as residual air were made before and after the administration of albumin intravenously in 5 patients.

*Metabolic results.* Table I summarizes the changes in concentration of the various plasma protein fractions and the alteration of nitrogen balance resulting from the infusion of albumin in 6 patients with pneumonia. The treated patients exhibited increments in albumin concentration of 0.98 to 1.61 g% following the administration of 100 to 170 g of albumin over 4 to 6 days, and in most instances these increments were maintained over the following week of observation. In contrast the controls showed but slight change in albumin levels. Furthermore, the treated patients retained more protein than could be accounted for by the amount of albumin

‡ The salt-poor human albumin was furnished through the kindness of Mr. R. B. Clark, by the Cutter Laboratories, Berkeley, Calif.

2 Zeldis, L. J., Alling, E. L., McCoord, A. B., and Kulka, J. P., *J. Exp. Med.*, 1945, **82**, 157.

§ Courtesy of Dr. H. R. Brown, Jr., of Department of Medicine.

alone. The magnitude of this protein retention is apparently dependent upon the dietary protein ingested, as well as the albumin administered. The patient (S.W.) who showed the least retention of protein, received only limited quantities of the special liquid diet for 2 days due to a misunderstanding of the orders. Contrariwise the patient (E.E.) who retained 598 g of protein during the period of observation was the one who requested and ingested more than the prescribed amount of the diet.

In addition to the changes in albumin concentration in the treated patients, there were reductions in the globulin fractions, especially the alpha-globulins (Table I). The mechanisms‖ of these alterations in the various fractions of the plasma proteins are not clear from these studies. Suitable isotope studies yielding accurate data on rates of protein turn-over will be needed to determine whether there is impaired synthesis or utilization, of the albumin and globulins under such conditions of stress.

*Cardio-respiratory results.* One patient, clinically considered to have chronic pulmonary disease, basilar pneumonia and shock, was given 25 g of albumin intravenously. Within 3 hours this patient developed acute pulmonary edema. Electrocardiogram showed left bundle branch block and ischemic type of T waves. Before definitive therapy could be instituted the patient died. Autopsy showed coronary arteriosclerosis, cardiac dilatation and hypertrophy, myocardial scars and pulmonary edema. Thus, the cause of the shock probably was due to coronary artery disease, and the administered albumin evidently contributed to pulmonary congestion. By electrophoresis the plasma albumin concentration was initially 2.0 g%. Because of this experience the secondary cardio-respiratory effects of albumin were investigated in all patients subsequently treated. Before any albumin therapy, each patient was found to have hypotension with systolic blood pressure ranging between 88 and 104 mm of mercury. Following the infusion of albumin, the effects were quite variable, even in the same patient on consecutive days. The trend of changes, however, were a slowing of the pulse together with increased blood pressure, stroke volume and cardiac output. The venous pressure often rose, but not above 12 cm of water. One patient (C. S.), who had had a myocardial infarction several months previously, showed no signs of cardiac dilatation or pulmonary congestion. The breath-holding time was often increased. Finally, the observed reductions in pulmonary volumes immediately after albumin therapy were indicative of pooling of blood within the lungs.

*Summary.* Six patients recovering from the acute febrile reaction of severe pneumococcic lobar pneumonia were given salt-poor human albumin intravenously. Electrophoretic analyses of the plasma proteins showed significant increases in the albumin concentration, together with reduction in globulins, particulary alpha-globulin, following this therapy. Simplified nitrogen balance studies showed retention of more protein than could be accounted for by the amount of albumin alone. Another patient with shock related to serious heart disease succumbed from pulmonary edema shortly after the administration of albumin. The secondary cardio-respiratory effects of albumin therapy were investigated in 5 patients.

‖ There was no significant increase in plasma volume by the Evans blue technic in the patient who received 150 g of albumin (the authors are indebted to Dr. L. A. Kohn for this observation). By urinary chromatography there was no significant change in renal excretion of amino acids in another patient treated with albumin. (The authors are indebted to Dr. C. Dent of Department of Pathology for this observation.)

16735

# Histochemical Demonstration of Sites of Phosphamidase Activity.*

G. Gomori.

*From the Department of Medicine, The University of Chicago, Chicago, Ill.*

In the course of experiments on the histochemical specificity of phosphatases the use of 18 out of 19 different substrates resulted in the production of identical patterns of enzyme distribution in all tissues and in all ranges of pH.[1] The nineteenth substrate, p-chloranilidophosphonic acid, included because of the existence of a specific "phosphamidase",[2-4] yielded a pattern identical with the others at pH 9 and 7, but produced strikingly different pictures at pH 5. Since this finding is a strong evidence in favor of the existence of a specific enzyme, a technic was developed for its histochemical demonstration.

*Experimental.* p-Chloranilidophosphonic acid (p-$ClC_6H_4NHPO(OH)_2$ was synthesized by the method of Otto.[5] The crude product was dissolved in an excess of $NH_4OH$, filtered, and neutralized, under ice cooling, with acetic acid. A slight excess of acetic acid was added (to about pH 4), and the solution was placed in the refrigerator. In a few hours the free phosphonic acid precipitated almost quantitatively. It was filtered under suction, washed with ice cold water, and dried *in vacuo*. The dry powder was extracted with cold absolute alcohol to remove p-chloraniline and dried. A 0.1 M stock solution was prepared by dissolving a calculated amount of the powder in an excess of 10% $NH_4OH$, adjusting the solution with dilute acetic acid to about pH 8 and filling it up to volume with distilled water. Such stock solutions were found to remain stable at refrigerator temperature for many weeks.

Thin slices of fresh tissue were fixed in a number of different fixatives, chilled acetone, 95% and absolute alcohol being found most satisfactory. Paraffin sections were incubated at 37°C from 2 to 48 hr in mixtures containing various amounts of substrate, Pb $(NO_3)_2$, and buffer in the range from pH 4.5 to 7.5. The best and most constant results were obtained at pH 5.4 to 5.8, the concentration of the substrate being 0.005 M, that of Pb $(NO_3)_2$, 0.0025 M and that of the buffer, 0.05 M. The presence of $MnCl_2$ in a concentration of 0.002 to 0.005 M greatly intensified the picture; Mg and Ca salts were without marked effect. Above pH 6.2 the reaction became distinctly weaker, disappearing altogether at pH 6.7. Above this pH level pictures corresponding to the distribution of nonspecific alkaline phosphatase were obtained. Below pH 5.3 confusing artifacts consisting in the Pb impregnation of various structures such as connective tissue and muscle fibers, nerves, mucin, etc. were often observed. These artifacts were present even in slides inactivated by dipping them for 5 minutes in Lugol's iodine solution prior to incubation, whereas true enzymatic reaction was completely abolished by this treatment. Since the substrate is not entirely stable around pH 5.6, the slides must be placed in the solution at an angle, the section facing downward, to avoid, as far as possible, the indiscriminate precipitation of Pb phosphate on the tissue. This maneuver will cause the precipitate to accumulate on the back surface of the slides from which it can be wiped off. Collodion coating of the slides interferes with

* This work has been done under grants from the Douglas Smith Foundation for Medical Research of The University of Chicago, and from the Pathology Study Section of the U. S. Public Health Service.

1 Gomori, G., Proc. Soc. Exp. Biol. and Med., 1949, to be published.

2 Walschmidt-Leitz, E., and Koehler, E., *Biochem. Z.*, 1933, **258**, 360.

3 Ichihara, M., *J. Biochem.* (Japan), 1933, **18**, 87.

4 Bredereck, H., and Geyer, E., *Z. physiol. Chem.*, 1938, **254**, 223.

5 Otto, P., *Ber. deutsch. chem. Ges.*, 1895, **28**, 617.

the reaction; in fact, if the tissues happened to be embedded through collodion the latter must be removed before incubation by passing the slides through an alcohol-ether mixture or acetone.

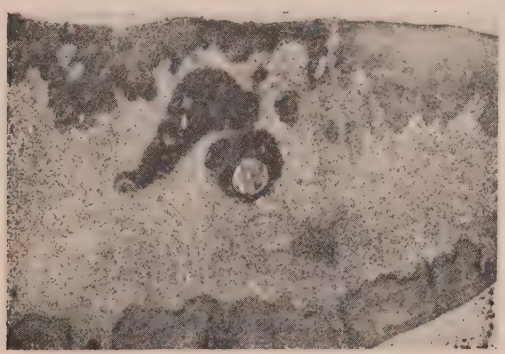


FIG. 1.
Squamous carcinoma of the cervic uteri. Note abrupt start of reaction at the edge of the malignant change.

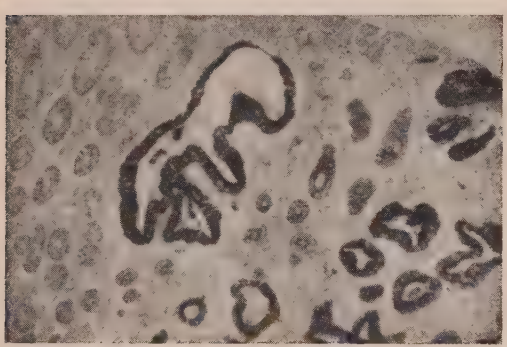


FIG. 2.
Adenocarcinoma of colon. Intense staining of carcinomatous glands; normal mucosa unstained.

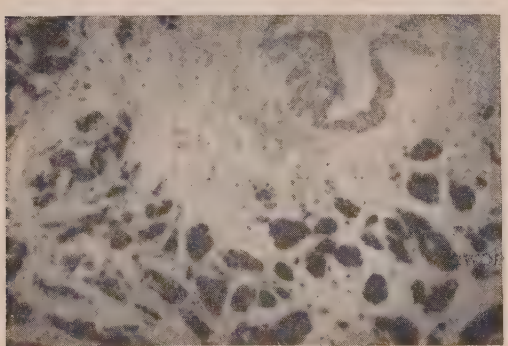


FIG. 3.
Scirrhous carcinoma of breast. Note unstained ducts.

The procedure to be described is recommended.

1. Incubate slides at 37°C for 10 to 24 hr in the following mixture:

To 50 ml of 0.05 M maleate buffer pH ± 5.6 (5.8 g of maleic acid + 62 ml of N NaOH in 1000 ml) add 1 to 1.5 ml of 0.1 M Pb $(No_3)_2$ solution and a few drops of a 10% solution of $MnCl_2$; shake until the initial precipitate dissolves. Add 2 ml of 0.1 M phosphonate stock solution. Place mixture in a 45 to 60°C oven for about 30 minutes until the turbidity consisting of $Pb_3(PO_4)_2$ settles. Filter mixture into a Coplin jar. Support latter in an inclined position (at an angle of about 30°); place slides in it with the section facing downward.

2. Rinse slides in distilled water. Wipe precipitate from backs of the slides and around the tissue.

3. Remove superficial precipitate by placing slides in a 0.1 M citrate buffer of pH 4.5 to 5. As soon as the slide appears to be completely clear around the tissue, rinse it thoroughly under the tap. This differentiation is the most critical step in the procedure since insufficient treatment of the section may leave a precipitate, appearing black in the finished section, scattered all over the slide while overtreatment may remove part or all of the enzymatic reaction product.

4. Treat slides with ammonium sulfide, etc., as in the method for lipase.[6]

*Results.* The dependability of the method in its present form, as far as uniformity of results is concerned, is not entirely satisfactory. Usually, long series of sections from various tissues will stain quite uniformly. Occasionally, however, partial or total failures are observed. Some sections may fail to stain altogether; others will show a patchy distribution of the reaction; still others may show a reaction at an abnormal site such as the nuclei instead of the cytoplasm. It is a curious fact that the reaction will remain selective for the same cell of group of cells, regardless of differences in its intracellular localization. Some but not all of these irregu-

[6] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 362.

larities may be caused by improper fixation since they may show up in only one or two amidst a large number of perfectly well stained consecutive serial sections. Nonspecific impregnations may also occur; they can be recognized as such by incubating inactivated slides (treated for 10 minutes with Lugol's solution or with any N mineral acid) as controls. The inactivated sections will show the same artifacts.

In successful slides, however, there is a typical localization of the reaction, varying somewhat with different species. The enzyme is present mainly in the cytoplasm (often in a granular form) but also in some of the nuclei. Moderate amounts are found in many tissues such as the liver, where its distribution often but not invariably follows that of glycogen; in the secretory portions of the renal tubules; in the adrenal cortex; in the small intestine where its localization is similar to that of alkaline phosphatase; in the epithelial lining of the bronchi; in the beta cells of the pancreatic islets of the mouse (not constantly); in the lachrymal gland of the hamster; and many others. However, a very intense reaction, much stronger than at the sites mentioned, is obtained in two tissues: the grey matter of the central nervous system (especially that of the cerebellum), and in malignant tumors. Twenty-four adenocarcinomas of the gastrointestinal tract, 4 squamous carcinomas, 2 hypernephromas and a number of other epithelial tumors such as embryonic carcinomas of the testis, cancers of the prostate, breast and bronchi, Walker rat carcinomas No. 256 and a butter yellow tumor stained very intensely and selectively. The reaction may start quite abruptly at the border of the malignant change, or the normal tissue may show a slight to moderate staining up to a distance of 1 or 2 mm from the edge of the neoplasm. In a few cases where the neoplasm showed various degrees of atypia in its different portions, the most atypical portions showed the most intense staining. So far not a single carcinoma has been found out of a total of 64 cases which did not show a sufficient reaction to set it off sharply against its environment. With sarcomas the results were much less constant. Some of them did not stain at all; others stained in an uneven, patchy way, and only a few stained as uniformly as carcinomas did. Two papillomas of the bladder, questionably malignant, stained moderately heavily. Granulomas such as sarcoidosis and human tuberculosis usually did not stain or showed a faint to moderate reaction in the giant cells and, rarely, even in the epitheloid cells. Tubercles in the guinea pig stained rather heavily.

*Summary.* A histochemical method for the demonstration of sites of phosphamidase activity is described. Small amounts of the enzyme are present in many normal tissues; large amounts are found in the grey matter of the central nervous system and in malignant epithelial tumors.

16736

## Inhibition of the Shwartzman Phenomenon by Local Application of Bromobenzene and Other Solvents.*

LEWIS THOMAS AND CHANDLER STETSON, JR.

*From the Department of Pediatrics, Johns Hopkins University School of Medicine, and the Harriet Lane Home, Johns Hopkins Hospital, Baltimore, Md.*

When an intradermal injection of toxin derived from certain Gram-negative microorganisms is followed, after a period of approximately 20 hours, by an intravenous injection of similar bacterial toxin, a gross hemorrhage involving the entire injected skin area occurs. This hemorrhagic reaction has become known as the Shwartzman phenom-

* This work was aided by a grant from the Life Insurance Medical Research Fund.

enon, in acknowledgement of the investigator who originally described it.[1]

During the course of recent studies on the mechanism of the Shwartzman phenomenon, it was found that the hemorrhagic reaction could be completely inhibited by the application of certain organic solvents to the prepared skin site, prior to the intravenous injection. It was also observed that these substances caused a marked increase in the permeability of the vessels of normal skin to circulating dye.

*Materials and methods.* Male white rabbits weighing from 1.8 to 2.5 kg, were used in all experiments. The entire abdomen was freed of hair by clipping and shaving at least 24 hours before the experimental day.

The bacterial toxins employed were 1) Meningococcal toxin No. 44B,† 2) a purified polysaccharide derived from culture filtrates of *Serratia marcescens,*‡ and 3) an "agar washings" filtrate prepared from cultures of *B. coli* according to Shwartzman's technic.[2]

Each bacterial toxin was titrated in 3 or more rabbits to determine its skin preparing potency. Similar titrations were carried out to ascertain the optimal amount of each preparation for intravenous provocation of the phenomenon. The dose of each toxin to be used intradermally in the following experiments was arbitrarily chosen as that amount which, when injected intradermally in a volume of 0.5 cc, resulted in satisfactory skin preparation of at least 75% of the rabbits tested. The intravenous dose of each agent was chosen as that amount, which, when injected intravenously in a volume of 2.0 cc, elicited positive reactions in at least 75% of satisfactorily prepared rabbits.

In testing the inhibitory property of locally applied substances, two skin areas were prepared by the intradermal injection of toxin, one on each side of the abdomen. These injections were spaced as far apart as possible, and the side used for testing inhibition was alternated in the rabbits of each experimental group in order to avoid the effect of possible differences in the natural susceptibility of the two sides of the abdomen.[2]

*Results.* The effect of a single application of bromobenzene was tested by applying this substance with a cotton swab over one side of the abdomen, at various times before and after an intradermal injection of bacterial toxin had been made on both sides. The intravenous injection of bacterial toxin was given 20 hours after the intradermal injection.

The inhibitory effect of bromobenzene is illustrated in Table I, in which it will be seen that when the local application was made at the same time as the intradermal injection of meningococcal toxin, or at various times thereafter up until the time of the intravenous injection, the Shwartzman phenomenon did not occur. Similar results were obtained in experiments using *S. marcescens* and *B. coli* toxins.

Maximum inhibition was brought about when bromobenzene was applied between 16 and 20 hours after skin preparation, as is indicated in Table I. When the skin was painted before the intradermal injection, no inhibition occurred; indeed, in a limited number of observations it appeared that the lesions in such areas tended to be more extensive than in unpainted control sites. When painting was delayed until 21 hours, or one hour after the intravenous injection, inhibition was not observed.

Various amounts of bromobenzene were dissolved in ether and tested 18 hours after skin preparation, in order to determine the concentration necessary for the effect. It was found that inhibition occurred regularly with 25 and 50% solutions, while the effect was less uniformly obtained with 5% and not at all with 1% solutions.

In subsequent experiments, other organic solvents were tested for similar inhibiting properties. Chlorobenzene, iodobenzene and benzene caused inhibition of the Shwartzman phenomenon, although the degree of inhibition was not as complete as with bromobenzene.

[1] Shwartzman, G., PROC. SOC. EXP. BIOL. AND MED., 1928, **25**, 560.

† Obtained from Dr. Gregory Shwartzman, and prepared by the method of this author.[2]

‡ Obtained from Dr. Murray Shear.

[2] Shwartzman, G., Phenomenon of Local Tissue Reactivity. Paul B. Hoeber, Inc., New York. 1937.

TABLE I.
Inhibition of the Shwartzman Phenomenon by Local Application of Bromobenzene at Various Intervals Before and After the Intradermal Injection of Meningococcal Toxin.

| Bromobenzene applied to skin | No. of rabbits | Shwartzman reaction* | | % inhibition |
|---|---|---|---|---|
| | | Bromobenzene treated side | Untreated side | |
| 24 hr before preparation | 4 | 4* | 4 | 0 |
| 4 " " " | 7 | 7 | 7 | 0 |
| At same time as preparation | 6 | 3 | 6 | 50 |
| 4 hr after " | 4 | 1 | 4 | 75 |
| 8 " " " | 4 | 1 | 4 | 75 |
| 16 " " " | 4 | 0 | 4 | 100 |
| 18 " " " | 6 | 0 | 6 | 100 |
| 20 " " " | 4 | 0 | 4 | 100 |
| 21† " " " | 3 | 3 | 3 | 0 |

* Figures refer to number of rabbits showing hemorrhagic reaction at indicated skin area following intravenous injection of meningococcal toxin. Latter injection given at 20 hours after skin preparation.

† These rabbits received intravenous toxin one hour before the application of bromobenzene.

Chloroform and methyl salicylate, when applied 20 hours after the intradermal injection, caused inhibition in a majority of animals, but these agents yielded less constant results when applied at earlier intervals. Ether, acetone and alcohol had no effect on the Shwartzman phenomenon. In order to test the effect of a substance with primary skin irritative effects but without solvent action, 10% formalin was applied in a series of rabbits; this material had no effect on the phenomenon.

Observations were made on the local reaction of normal rabbit skin to single and repeated applications of bromobenzene. Within 30 to 60 minutes after a single painting, the skin exhibited a slight erythema. During the next few hours some edema occurred in the painted area in many of the animals. After 12 hours the skin usually appeared normal. However, after several days some superficial flaky desquamation of the skin commonly occurred. It was of incidental interest that the regrowth of hair on the shaven abdomen occurred more rapidly during the following weeks in areas which had been painted with bromobenzene. The effect of repeated painting with bromobenzene was similar to that following a single application, but the edema and desquamation were more extensive.

It is known that irritation of normal skin may cause an increase in local permeability to circulating dyes.[3] The effect of bromobenzene on permeability was tested by applying this substance to the skin of normal rabbits which were simultaneously given an intravenous injection of 5 cc of 5% Evans Blue Dye (T-1824). It was found that within 5 minutes after painting with bromobenzene, the painted area became deeply stained with dye. The local accumulation of dye was sharply confined to the painted area and outlined almost exactly the extent of application of the swab. The skin remained deeply stained for about 12 hours, after which fading occurred gradually.

Before testing the effect of bromobenzene on the permeability of the vessels in skin tissue which had been prepared for the Shwartzman phenomenon, it was of interest to determine whether such tissues showed any increase in permeability to Evans Blue dye. Accordingly, rabbits were given intradermal injections of meningococcal toxin and at various intervals thereafter were given an intravenous injection of 5 cc of 5% Evans Blue. It was observed that the prepared skin did not become stained at any time up until the actual precipitation of hemorrhage following the intravenous injection of toxin. These results were similar to those described by Bordet,[4] who used trypan blue dye in similar experiments.

[3] Menkin, V., Dynamics of Inflammation. The Macmillan Co., New York. 1940.

[4] Bordet, P., *Ann. Inst. Pasteur*, 1936, **56**, 325.

Having ascertained that skin areas with meningococcal toxin were not more permeable to circulating dye than normal rabbit skin, the effect of bromobenzene on prepared skin was determined. The results were quite different from those seen in normal skin. When bromobenzene was applied to an area which had been injected intradermally with toxin eighteen hours previously, and an intravenous injection of Evans Blue given simultaneously, no dye appeared in the prepared area. When a large surface of the abdomen of a prepared rabbit was painted, the contrast between the deep blue staining of the surrounding normal skin and the pink, unstained prepared area was striking.

These observations suggested that the vessels in a prepared skin area were actually less permeable to dye than normal vessels, or were less responsive to the permeability-enhancing property of bromobenzene. Similar results were obtained with surface applications of chlorobenzene, iodobenzene, and benzene, and to a less striking degree with chloroform and methyl salicylate; in each instance normal skin showed staining with the dye while prepared skin showed little or no staining.

*Discussion.* The inhibition of the Shwartzman phenomenon by the local application of bromobenzene and certain other organic solvents was a somewhat accidental finding. As a result of certain other experimental data,[5] an hypothesis was entertained which implicated sulfhydryl-activatable tissue proteolytic enzymes, or "cathepsins", in the mechanism of damage to blood vessel walls in the Shwartzman phenomenon. Crabtree[6] demonstrated that the application of bromobenzene caused prompt reduction in the amount of gluthathione in skin tissue, presumably as the result of local detoxification of bromobenzene. Accordingly, the possibility was considered that an application of bromobenzene to a skin area which had been prepared for the Shwartzman phenomenon might inhibit the hemorrhagic reaction by interfering with the activation of tissue protease. The finding that inhibition of the phenomenon was, in fact, produced by bromobenzene does not indicate that this is the mechanism by which the inhibition took place, in view of the observation that other unrelated substances such as chloroform and methyl salicylate caused a similar effect.

The capacity of bromobenzene, as well as the other solvents tested, to bring about a local increase in the permeability of normal skin to circulating Evans Blue dye offers a second possible explanation for the inhibitory action of these substances on the Shwartzman phenomenon. Although no increased permeability was demonstrable in prepared skin tissue by the dye method, it is reasonable to assume that a similar change, although less in degree, may have occurred. Under such a circumstance, inhibition of the Shwartzman phenomenon may have been caused by the advent of an inhibitory substance from the blood into the prepared area, or by the departure of a damaging substance from the prepared area.

The third possible explanation to be considered is that the inhibition is the result of a wholly non-specific damaging action of bromobenzene on the skin blood vessels, interfering with their reactivity to a variety of other stimuli.

The question of the permeability of skin capillaries is of importance in considering the pathogenesis of the Shwartzman phenomenon. If these vessels were more permeable than normal, as is reported to be the case in other varieties of inflammatory tissue,[3] the accumulation of toxic material from the circulating blood would require consideration as a possible mechanism for the tissue damage,[3,7] even though the likelihood of bacterial toxin having a direct hemorrhagic effect in such small concentrations seems remote. The studies of blood vessel permeability with intravenously injected Evans Blue dye indicate that the prepared skin is not more permeable to this material, and the results obtained with bromobenzene suggest that the vessels are less permeable, at least to this type of permeability-enhancing stimulus.

[5] Thomas, L., and Stetson, C. A., in press.

[6] Crabtree, H. G., *Cancer Research*, 1944, **4**, 688.

[7] Moritz, A. R., *J. Exp. Med.*, 1937, **66**, 603.

Other studies in this laboratory[5] have shown that skin tissue prepared for the Shwartzman phenomenon exhibits an abnormal degree of aerobic glycolysis and lactic acid accumulation. Whether this alteration is related to an impairment in the permeability of vessels in prepared skin is a subject for further investigation. It is of interest to note that the application of bromobenzene to prepared skin had no demonstrable effect on the degree of aerobic glycolysis exhibited by such tissue.[8]

*Summary.* Complete inhibition of the Shwartzman phenomenon was produced by a single application of bromobenzene to the surface of prepared skin areas at any time during the 20 hours after the intradermal injection of bacterial toxin. Similar results were obtained with other benzene derivatives, and, less constantly, with chloroform and methyl salicylate.

Areas of rabbit skin which were prepared by the intradermal injection of meningococcal toxin showed no increase in permeability to Evans Blue dye, when the dye was injected intravenously.

A single application of bromobenzene to the surface of normal rabbit skin resulted in the prompt appearance of intravenously injected Evans Blue dye in the painted area. In contrast, little or no dye appeared in painted areas which had previously been injected with meningococcal toxin. Similar results were obtained when skin was painted with other benzene derivatives, chloroform and methyl salicylate.

The possible bearing of these observations on the problem of the mechanism of the Shwartzman phenomenon is discussed.

8 Unpublished observation.

16737

## Influence of Dinitrophenol on Body Temperature Threshold for Thermal Polypnea.*

V. E. HALL, F. P. ATTARDO, AND J. H. PERRYMAN.

*From the Department of Physiology, Stanford University Medical School, Calif.*

The capacity of dinitrophenol (DNP) to cause hyperthermia in animals through accelerating heat production is well known. In animals at low environmental temperatures, however, it reduces body temperature and total heat production,[1] apparently through reducing the activity of the cold defense mechanism, as shown by depression of shivering.[2] In discussing the mechanism of this inactivation of cold defense, Hall, Crismon and Chamberlin[2] suggested two possibilities: (a) DNP might depress the centers involved in cold defense by a nonspecific toxic action similar to that by which it depresses other nervous processes such as spontaneous running activity;[3] (b) DNP, which increases the oxygen consumption of cerebral cortical tissue in concentrations likely to be attained when ordinary doses are injected,[4] might accelerate the metabolic activity of the temperature regulating centers, so causing the centers to behave as if heated which would reduce or abolish shivering. If the latter were the case, one would expect from the known reciprocal relationship of the cold and heat defense mechanisms that, when the body temperature

* This work was done under a contract between the Air Materiel Command, Wright Field, and Stanford University.

1 Giaja, J., and Dimitrijevic, I. N., *Arch. internat. Pharm. Therap.*, 1933, **45**, 342; Tainter, M. L., *J. Pharm. Exp. Therap.*, 1934, **51**, 45.

2 Hall, V. E., Crismon, J. M., and Chamberlin, P. E., *J. Pharm. Exp. Therap.*, 1937, **59**, 193.

3 Hall, V. E., and Lindsay, M., *J. Pharm. Exp. Therap.*, 1934, **51**, 430.

4 Fuhrman, F. A., and Field, J., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 504.

TABLE I.

Effect of Dinitrophenol and of Salt Solution on Threshold for Thermal Polypnea of Rabbits.

| Rabbit No. | Normal threshold °C | DNP threshold °C | Change in threshold °C |
|---|---|---|---|
| | A. Dinitrophenol (20 mg per kg) | | |
| 1A | 39.4 | 41.0 | +1.6 |
| 1B | 40.1 | 41.0 | +0.9 |
| 2A | 40.4 | 41.9 | +1.5 |
| 2B | 40.9 | 40.7 | —0.2 |
| 2C | 40.9 | 41.4 | +0.5 |
| 3A | 40.0 | 40.9 | +0.9 |
| 3B | 39.5 | 40.2 | +0.7 |
| 4A | 40.3 | 41.7 | +1.4 |
| 4B | 40.1 | 41.6 | +1.5 |
| 4C | 40.1 | 41.0 | +0.9 |
| Avg | 40.2 | 41.1 | +0.9 |
| | B. Controls with physiological salt solution | | |
| Rabbit No. | Normal threshold | Threshold after saline | Change in threshold |
| 5A | 39.5 | 39.1 | —0.4 |
| 5B | 39.0 | 39.0 | 0.0 |
| 5C | 39.1 | 39.6 | +0.5 |
| 6A | 39.0 | 38.1 | —0.9 |
| 6B | 38.4 | 38.3 | —0.1 |
| Avg | 39.0 | 38.8 | —0.2 |

is raised, processes accomplishing heat loss should be more readily activated under the influence of DNP than in its absence. Among other effects, DNP should (at constant environmental temperature) reduce the body temperature level at which thermal polypnea appears. The experiments described here were designed to test this possibility.

Albino rabbits weighing between 2 and 3 kg were placed in a box in which the air temperature was maintained at 30°C ± 2°). This environmental temperature is higher than that at which the rabbit can maintain a constant body temperature. Accordingly a progressive rise in body temperature occurred, and with it a rise in respiratory rate. When the latter reached 300 per min., the rectal temperature was measured with a mercury thermometer. This value was designated as the normal threshold for thermal polypnea.

The rabbits were then placed in a refrigerator at about 10°C per 45 to 60 min. Their rectal temperatures were reduced by this procedure to values well below the normal threshold for thermal polypnea. A dose of 20 mg per kg of the sodium salt of 2,4-dinitrophenol was then injected intramuscularly and the rabbits returned to the box at 30°C. As their rectal temperatures rose under the combined action of the high environmental temperature and the calorigenic action of the drug, the respiratory rate accelerated. The rectal temperature at which it again reached 300 per min. was determined and designated as the DNP threshold for thermal polypnea.

The results of 10 such experiments are shown in Table I. With one exception, the DNP thresholds for thermal polypnea are greater than the corresponding normal thresholds. The average change in threshold after DNP was +0.9°C. The chances that these results could have occurred by chance have been calculated to be less than one in one thousand.

A second series of 5 rabbits was treated in the same manner except that a volume of physiological salt solution equal to the volume of DNP solution was injected. The results, also given in Table I, show an average reduction of the threshold determined after saline injection amounting to 0.2°C. This finding rules out the possibility that, in the DNP series, the elevated threshold might be due to factors other than those attributable to DNP itself.

In view of the possibility that the dose of 20 mg per kg might have toxic effects not shown by lower doses, 3 rabbits subjected to

the procedure described above, were given doses of 10 mg per kg. The rectal temperatures at which respiratory rates of 200 per min. were attained before and after DNP respectively were: 39.8 and 40.2; 39.8 and 40.0, and 39.6 and 39.8°C, the threshold being increased by an average of 0.3°C. This difference is smaller than but in the same direction as the difference obtained with the larger dose of DNP.

*Conclusion.* We may conclude that DNP in doses of 10 and 20 mg per kg, although it does not prevent the occurrence of thermal polypnea, does significantly increase the rectal temperature level at which such polypnea appears. It accordingly appears that DNP does not sensitize the heat defense mechanism as would be required by the second hypothesis stated above. Rather it suggests that DNP depresses both heat and cold defense mechanisms by a nonspecific toxic action.

16738

## Effect of Insulin on Rate of Metabolism of Ethyl Alcohol.

HENRY W. NEWMAN AND WINDSOR C. CUTTING.

*From the Departments of Medicine and of Pharmacology and Therapeutics, Stanford University School of Medicine, San Francisco, Calif.*

A review of the literature shows that the great majority of workers who have investigated the problem found that insulin in adequate dosage was effective in accelerating the rate of metabolism of alcohol. Thus Supniewski[1] showed that 1 unit of insulin per kg administered to rabbits about doubled the rate at which alcohol disappeared from the blood. Newman and Cutting[2] gave the same dose of insulin to 2 human subjects after administration of alcohol intravenously and found a 50% increase in rate of metabolism. Serianni[3] found a similar increase in man after a dose of 20 units of insulin per individual. Widmark[4] showed that 0.85 unit per kg in the dog accelerated the rate of alcohol metabolism, particularly in dogs with an initially low rate of metabolism of alcohol, but that the effect varied in intensity from dog to dog and in the same dog from day to day. Clark and his associates[5,6] gave doses of 1 and 2 units per kg to dogs and found a significant increase in rate of disappearance of alcohol from the blood.

Gregory, Ewing and Duff-White,[7] on the contrary, reported that "there is no evidence from 24 experiments in 6 dogs that insulin, glucose, or insulin plus glucose increases the rate of metabolism of ethyl alcohol." They refer to certain reports in the literature in support of this finding. Thus Herschfelder and Maxwell[8] concluded that insulin in doses up to 3 units per kg did not expedite the disappearance of symptoms of intoxication in the rabbit. When one considers that such doses of insulin might well in themselves induce a state similar to alcoholic intoxication in its symptomatology, one is not justified in drawing any conclusions from these experiments regarding the effect of insulin on rate of alcohol metabolism. Fleming and Rey-

1 Supniewski, J., *J. Biol. Chem.*, 1926, **70**, 13.

2 Newman, H. W., and Cutting, W. C., *J. Clin. Invest.*, 1935, **14**, 945.

3 Serianni, E., *R. C. Accad. Lincei*, 1935, **21**, 394.

4 Widmark, E. M. P., *Biochem. Z.*, 1935, **282**, 79.

5 Clark, B., and Morrissey, R., *Am. J. Physiol.*, 1938, **123**, 37.

6 Clark, B., Morrissey, R., Fazekas, J., and Welch, C., *Quart. J. Studies Alc.*, 1941, **1**, 663.

7 Gregory, R., Ewing, P., and Duff-White, V., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 206.

8 Hirschfelder, A. D., and Maxwell, H. C., *Am. J. Physiol.*, 1924, **70**, 520.

nolds[9] gave one human subject a dose of 10 units of insulin and found no effect on the rate of disappearance of alcohol injected intravenously. Here the dosage is conspicuously lower than that employed by those workers cited above who found an effect from insulin. The work of Goldfarb, Bowman and Parker[10] can hardly be classed as supportive, since they found that while neither insulin nor glucose alone expedited the removal of alcohol from moderately intoxicated patients, a combination of the two was effective. Neither can the unelaborated statement of Mirsky and Nelson[11] that in dogs "treatment with insulin and glucose" had little effect on the rate of alcohol metabolism be very heavily weighted as evidence. Thus it is seen that the evidence in the literature referred to by Gregory and coworkers is not impressive. However, a short communication by Harger and Hulpieu[12] is less easily disposed of. These workers found that the rate of fall of alcohol concentration in dogs after gastric administration of doses from 1.5 to 3.0 g per kg was not changed by the administration of 1 unit of insulin per kg, alone or with glucose. Gregory and coworkers would, however, object to this work on the basis that the alcohol was given by mouth, since they reported that after gastric administration the blood alcohol levels were so variable, and disappearance curves so irregular, that this was an unsatisfactory method of giving alcohol for this type of investigation.

For this reason, they[7] administered the alcohol intravenously, and followed the concentration in the blood until it approached zero. They published one graph which shows the curves of blood alcohol concentration in one dog after a control dose of 2.0 cc per kg of alcohol alone, alcohol and glucose, alcohol and insulin 1 unit per kg, and finally alcohol and combination of insulin and glucose. All of the curves reached the base line in from 11 to 12 hours, so that there is no evidence in this dog of acceleration by insulin. The authors stated that the results in the other 5 dogs were "practically identical", but did not publish the data on which they based this statement.

In view of the conflict of these results with most of the reported work, including our own work in man where the method of procedure was practically identical, it was felt that further investigation of the problem, adhering closely to the methodology of Gregory and coworkers, was indicated.

To this end, alcohol was administered intravenously to each of three dogs in a dose approximating 2 cc per kg, and the blood alcohol concentration followed with serial samples until it approached zero. Analyses for alcohol were carried out according to the method of Newman.[13] Extrapolation of the best-fitting line between these points to the base line was used to estimate the time required for disappearance of alcohol, and from this it was a simple matter to calculate the average rate of metabolism in mg per kg per hour. In order to establish fully the range of variation in this rate in a given dog from day to day, 3 controls with alcohol alone were run

TABLE I.
Rate of Metabolism of Alcohol With and Without Insulin in Dosage of 1 Unit per Kg Body Weight.

| Dog | Wt kg | Dose alcohol g/kg | Rate of metabolism mg/kg/hr Control | Insulin |
|---|---|---|---|---|
| 1 | 23 | 1.5 | 102 | |
| | | 1.5 | 98 | |
| | | 1.5 | 103 | |
| | | 1.5 | | 143 |
| 2 | 21 | 1.5 | 123 | |
| | | 1.5 | 102 | |
| | | 1.5 | 107 | |
| | | 1.5 | | 158 |
| | | 1.5 | | 164 |
| 5 | 10.5 | 1.43 | 102 | |
| | | 1.43 | 104 | |
| | | 1.43 | 106 | |
| | | 1.43 | | 112 |

[9] Fleming, R., and Reynolds, D., *J. Pharmacol. and Exp. Therap.*, 1935, **54**, 236.

[10] Goldfarb, W., Bowman, K. M., and Parker, S., *J. Clin. Invest.*, 1939, **18**, 581.

[11] Mirsky, I. A., and Nelson, N., *Am. J. Physiol.*, 1939, **127**, 308.

[12] Harger, R. N., and Hulpieu, H. R., *J. Pharmacol. and Exp. Therap.*, 1935, **54**, 145.

[13] Newman, H. W., *J. Pharmacol. and Exp. Therap.*, 1936, **56**, 278.

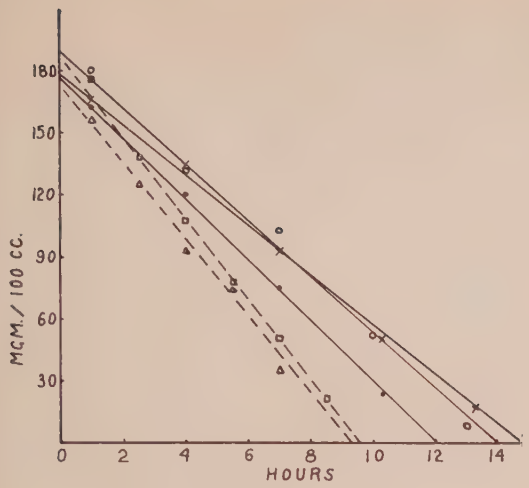



FIG. 1.

Blood alcohol concentrations after intravenous injection of 1.5 g of alcohol per kg in a dog. Three control injections are represented by crosses, open circles, and dots. Two injections with insulin 1 unit per kg are shown with open squares and open triangles. The best fitting lines between these points are extrapolated to the base line to estimate the time of disappearance of alcohol from the blood stream.

on each dog. Then the same procedure was repeated after administration of a single dose of 1 unit of regular insulin per kg, and in the animal which showed the greatest acceleration the insulin run was repeated so that we could be doubly sure of the results. The results are presented in Table I, and those in the dog which had the 2 trials with insulin are shown graphically in Fig. 1. It is seen that 2 of the 3 animals responded to this dose of insulin by an increase in rate of alcohol metabolism of approximately 50%, while the slight increase in the third animal, although deviating from the average of the control values by a considerable margin over the range of these values, was far less striking. The same procedure was repeated with two other dogs, with the exception that the dose of insulin was reduced to 0.5 unit per kg. In neither animal did this smaller dosage of insulin produce any acceleration of alcohol metabolism.

*Summary.* Insulin in a dose of 1 unit per kg was found to be variably effective in accelerating the rate of alcohol metabolism, the effect being striking in 2 dogs, much less in another. This is in accord with the variable results reported by Widmark.[4] Half this dose was entirely ineffective in 2 dogs. The failure of Gregory and coworkers[7] to demonstrate this accelerating action of insulin in adequate dosage must be due to the possibility that their 6 dogs fell, by chance, into the group of animals which does not show a striking acceleration with insulin.

16739

## Prolongation of Pseudopregnancy by Deciduomata in the Rat.*

B. M. PECKHAM AND R. R. GREENE.

*From the Departments of Physiology and Obstetrics and Gynecology, Northwestern University Medical School, Chicago, Ill.*

Astwood and Greep[1] have stated that the presence of deciduomata in the rat does not prolong the diestrus of pseudopregnancy. Ershoff and Deuel,[2] however, have presented data showing pseudopregnancy in the rat to be prolonged as much as 7 days by production of deciduomata. More recently Kamell and Atkinson[3] reported that deciduomata do not prolong pseudopregnancy in the mouse. The following data confirm those of Ershoff and Deuel.

* Supported in part by a grant from Ciba Pharmaceutical Products, Inc.

1 Astwood, E. B., and Greep, R. O., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 713.

2 Ershoff, B. H., and Deuel, H. J., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 167.

3 Kamell, S. A., and Atkinson, W. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 415.

TABLE I.
Prolongation of Pseudopregnancy by Deciduomata.

| Group | No. of rats | No. of deciduomata | Pseudopregnancy (days) | Standard error | St. error of diff.* and probability |
|---|---|---|---|---|---|
| Pseudopregnant | 18 | 8 | 13.33 | 0.220 | ±1.043 and <0.01 |
| Pseudopregnant with deciduomata | 10 | 8 | 20.60 | 1.287 | |
| Pseudopregnant with deciduomata | 11 | 4 | 18.55 | 1.290 | ±1.340 and <0.01 |
| Pseudopregnant with deciduomata removed | 17 | 4 | 14.25 | 0.680 | |

* Pooled variance. Snedecor *Statistical Methods*, University of Iowa College Press, 1947.

*Methods and results.* Daily vaginal smears were made from 18 female albino rats (150 to 250 g) of mixed strain. On the day of vaginal estrus (fully cornified smear) their cervices were stimulated electrically† to induce pseudopregnancy. Vaginal smears were made until the next vaginal estrus. The first day of diestrus was considered day one and the first day of full vaginal cornification the last day of pseudopregnancy. The average length of pseudopregnancy was found to be 13.33 days. (Table I).

Ten similarly prepared rats were subjected to laparotomy on the fourth day of pseudopregnancy. Both uteri were stimulated by passing a suture transversely through the walls and lumen at 4 points in each, tying loosely and leaving the suture *in situ* as a marker. On the 8th day of pseudopregnancy at a second laparotomy, the number of deciduomata formed in each rat was determined. Daily smearing was continued as before. Each rat responded with eight deciduomata, the maximum number possible. The average length of pseudopregnancy in this group was 20.60 days (see Table I). The difference of 7.27 days is highly significant statistically.

To determine whether some factor other than the presence of deciduomata was responsible for prolongation of diestrus an additional 28 pseudopregnant rats were treated as in the previous experiment except that at laparotomy on the 4th day *only one uterus (the right) was stimulated in 4 places.* On the eighth day the number of deciduomata formed in each was noted. Again all 28 rats responded with the maximum number, 4 deciduomata. The right uterus containing the deciduomata was then removed in 17 of the 28 rats. The average length of pseudopregnancy in the 17 rats in which the deciduomata were removed was 14.25 days (not significantly different from the length of normal pseudopregnancy). The length of pseudopregnancy in the 11 rats in whom the deciduomata were not removed was 18.55 days (not significantly different from the length of pseudopregnancy in the rats with 8 deciduomata).

As may be seen in Table I the difference of 4.30 days is statistically significant.

Our data demonstrate that the presence of deciduomata in the pseudopregnant rat significantly prolongs the period of diestrus. The mechanism involved is not known but the evidence presented here is not adequate to prove that it is a specific effect of the deciduoma, *per se.*

*Summary.* The presence of deciduomata prolongs the length of pseudopregnancy in the rat an average of 7.27 days beyond the normal duration of 13.33 days.

† A biopolar electrode attached to a Harvard inductorium was inserted in the vagina.

16740

# Effect of Cardiac Drugs on the Phosphorylated Intermediates of the Rat.*

T. E. Kimura. (Introduced by E. M. K. Geiling.)

*From the Department of Pharmacology, University of Chicago, Chicago, Ill.*

Reports[1-3] have appeared in the literature on the effects of cardiac drugs on glycogen metabolism of laboratory animals. Several studies on the effect of cardiac drugs on the concentration of certain acid-soluble phosphorus compounds have been reported. These findings have been summarized in a recent review[4] on the biochemistry and pharmacology of the heart. Wollenberger[5] concluded from his studies on dog heart-lung preparations that the primary action of cardiac glycosides must be concerned with a phase of myocardial contraction other than the generation of utilizable chemical energy. On a series of perfusion studies of the isolated hearts of cats, rabbits, and rats, with digitoxin, Sjoerdsma *et al.*[6] reported that the appearance of creatinine in the perfusion fluid is not altered. However, no systematic analysis of the phosphorylated intermediates of glycolysis has been reported. The purpose of the present investigation was to measure the concentration of a number of phosphorylated intermediates of glycolysis under various conditions of treatment with cardiac drugs.

*Experimental.* Normal Sprague-Dawley rats of approximately 250 g were digitalized intraperitoneally by the daily administration of 12 mg/kg of crystalline digitoxin in propylene glycol for 3 days. Control animals received equivalent amounts of the solvent throughout the digitalization period. Following a fasting period of 24 to 36 hours, the animals were anesthetized by the intraperitoneal injection of 45 mg/kg of sodium pentobarbital, and decapitated immediately after light anesthesia. The tissues were quickly blotted on filter paper and frozen between slabs of dry ice, weighed, and the phosphorus fractions then measured according to the methods outlined by LePage and Umbreit.[7] Glycogen in normal and digitalized rat heart was determined on separate samples of tissues by the method of Good *et al.*[8] The method of Folin and Malmros[9] was used for measuring the glucose after hydrolysis of glycogen.

None of the digitalized animals elicited any symptoms of over-digitalization, such as tremors or convulsions. The data presented in Table I indicate that digitoxin produced no marked alteration in the distribution of the acid-soluble phosphorylated intermediates of the rat heart. Although reports have appeared in the literature on changes in the phosphocreatine content of the heart under the influence of cardiac drugs, no such effect was noticed, under the conditions of the experiment.

Results presented in Table II indicate that there was no marked difference in heart glycogen values, but that there was an increased amount of liver glycogen in the digitalized animals as compared with the controls, there

* This work was supported by grants from the Life Insurance Medical Research Fund. The digitoxin used in these experiments was kindly supplied by Dr. K. K. Chen of the Lilly Research Laboratories, Indianapolis, Ind.

1 Liebig, H., *Arch. exp. Path. Pharmakol.*, 1940, **196**, 137.

2 Cherkes, A. I., *Acta Med., U.R.S.S.*, 1940, **3**, 155.

3 Bomskov, C., *Arch. exp. Path. Pharmakol.*, 1941, **198**, 232.

4 Chen, G., and Geiling, E. M. K., *Schweiz. Med. Wochenschrift*, Basle, 1947.

5 Wollenberger, A., *Am. J. Physiol.*, 1947, **150**, 733.

6 Sjoerdsma, A., Kun, E., Schueler, F. W., and DoValle, J. E., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 144.

7 LePage, G. A., and Umbreit, W. W., Manometric Techniques, Minneapolis, 1945.

8 Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 484.

9 Folin, C., and Malmros, H. J., *J. Biol. Chem.*, 1929, **83**, 115.

TABLE I.
Distribution of Acid-Soluble Phosphorus Compounds in the Heart of Normal and Digitalized Rats.

| Phosphorylated Intermediates* | Normal | | Digitalized | |
|---|---|---|---|---|
| A. Analysis on T.C.A. Extracts: | | | | |
| 1. "True" Inorganic Phosphorus | 993 | | 1100 | |
| 2. Phosphocreatine Phosphorus | 262 | | 293 | |
| 3. Phosphopyruvic | 1450 | | 1242 | |
| 4. Total Phosphorus | 3110 | | 3184 | |
| | Barium-Soluble | | Barium-Insoluble | |
| | Normal | Drug | Normal | Drug |
| B. Analysis of Fractions: | | | | |
| 1. Inorganic Phosphorus | 239 | 245 | 1232 | 1326 |
| 2. ATP 7 min Hydrolyzable | | | 229 | 200 |
| 3. Total Phosphorus | 877 | 1180 | 1693 | 2155 |
| 4. Fructose | 17 | 18 | | |
| 5. Fructose 1-6 Diphosphate | | | 102 | 97 |

* Expressed as micromoles/100 g.

TABLE II.
Effect of Digitoxin on Liver and Heart Glycogen of Rat Given 12 mg/kg Digitoxin in Propylene Glycol for 3 Days, Fasted 24-36 Hours, and Anesthetized with 45 mg/kg Sodium Pentobarbital.

| Tissue | Control | | | Digitalized | | |
|---|---|---|---|---|---|---|
| | Rat No. | % glycogen | Mean | Rat No. | % glycogen | Mean |
| Heart | 1 | 0.34 | | 4 | 0.30 | |
| | 2 | 0.27 | 0.27 | 5 | 0.29 | 0.30 |
| | 3 | 0.21 | | 6 | 0.31 | |
| Liver | 1 | 1.96 | | 4 | 2.06 | |
| | 2 | 1.88 | 1.85 | 5 | 2.32 | 2.39 |
| | 3 | 1.71 | | 6 | 2.81 | |

being 2.39% glycogen in the former and 1.85% in the latter. These values suggested the possibility that digitoxin may exert a protective action on liver glycogen by decreasing the rate of glycogenolysis and experiments were, therefore, conducted to measure this possibility. Samples of liver tissue from both digitalized and normal animals were allowed to stand at room temperatures for periods of 2, 5, and 8 minutes prior to freezing with dry ice. Results indicated that there was no difference in the rate of breakdown of glycogen in the excised liver tissue in normal and digitoxin treated rats. It should be noted that these and subsequent experiments, in which the liver has been removed from its normal environment, do not take into consideration the possible influence of epinephrine or insulin on glycolysis or glycogenesis *in situ*.

Inasmuch as Yorimitsu[10] had demonstrated that digitalis preparations promoted resynthesis of glycogen from lactic acid, experiments were conducted to ascertain the effect of digitoxin on the synthesis of liver glycogen from glucose in rat liver, using the method as outlined by DuBois *et al.*[11] Normal Sprague-Dawley rats of approximately 250 g were digitalized intraperitoneally with 5 mg/kg of crystalline digitoxin in propylene glycol for 3 days. Controls received equivalent amounts of the solvent during the digitalization. The experiments were begun following a fasting period of 24 to 36 hours. Data given in Table III show that the initial glycogen values were higher in the digitalized animals, and there was an indication of an increased amount of glycogenesis from injected glucose

[10] Yorimitsu, T., *Osaka Igakkai Zassi*, 1940, **39**, 1381.

[11] DuBois, K. P., Holm, L. W., and Doyle, W. L., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 102.

TABLE III.

Effect of Digitoxin on Synthesis of Liver Glycogen in Rats Given 5 mg/kg Digitoxin in Propylene Glycol for 3 days; Fasted 24 Hours; No Further Injection of Drug Given During Glycogenesis; No Convulsions or Tremors.

| | Control | | | | Digitalized | | | |
|---|---|---|---|---|---|---|---|---|
| Glucose schedule | Exp. No. | Rat No. | % glycogen | Mean | Exp. No. | Rat No. | % glycogen | Mean |
| No Glucose | I | 1 | 0.59 | | I | 9 | 0.95 | |
| | | 2 | 0.63 | 0.61 | | 10 | 0.85 | 0.90 |
| | II | 17 | 0.19 | | II | 25 | 1.02 | |
| | | 18 | 0.41 | 0.30 | | 26 | 0.99 | 1.00 |
| 0.15 g/hr for 2 hrs before death | I | 3 | 1.22 | | I | 11 | 2.36 | |
| | | 4 | 1.27 | 1.24 | | 12 | 2.52 | 2.44 |
| | II | 19 | 0.48 | | II | 27 | 1.86 | |
| | | 20 | 0.61 | 0.54 | | 28 | 1.43 | 1.64 |
| 0.15 g/hr for 4 hrs before death | I | 5 | 2.09 | | I | 13 | 2.81 | |
| | | 6 | 2.03 | 2.06 | | 14 | 2.71 | 2.76 |
| | II | 21 | 1.03 | | II | 29 | 1.96 | |
| | | 22 | 1.18 | 1.10 | | 30 | 2.80 | 2.38 |
| 0.15 g/hr for 6 hrs before death | I | 7 | 2.05 | | I | 15 | 2.86 | |
| | | 8 | 2.13 | 2.09 | | 16 | 2.84 | 2.85 |
| | II | 23 | 1.06 | | II | 31 | 2.86 | |
| | | 24 | 1.32 | 1.19 | | 32 | 2.43 | 2.64 |

TABLE IV.

Effect of Digitoxin on Synthesis of Liver Glycogen in Over-Digitalized Rats Given 4 mg/kg Digitoxin in Propylene Glycol for 3 days; Fasted 24 Hours; Additional Injection of 5 mg/kg Digitoxin Given at 0 Hour.

| | Control | | | Digitalized | | |
|---|---|---|---|---|---|---|
| Glucose schedule | Rat No. | % glycogen | Mean | Rat No. | % glycogen | Mean |
| No glucose | 34 | 0.31 | | 40* | 0.21 | |
| | 35 | 0.29 | 0.30 | 41* | 0.17 | 0.19 |
| 0.15 g/hr for 2 hrs before death | 36 | 1.01 | | 42† | 0.04 | — |
| | 37 | 0.86 | 0.93 | 43* | 0.29 | — |
| 0.15 g/hr for 4 hrs before death | 38 | 1.83 | | 44† | Died | — |
| | 39 | 1.73 | 1.78 | 45† | 0.02 | — |

* Occasional tremors.
† Tremors and convulsions.

in digitalized animals. There was no additional injection of the drug during the 6 hour period of glucose injection. None of the digitalized animals showed any tremors, convulsions, or other toxic symptoms. Difference in normal values of Experiment I and II are probably due to differences in amount of previously ingested food, prior to fasting.

The results given in Table IV show the effect of digitoxin on glycogen synthesis by the liver after administration of glucose to fasted over-digitalized rats. A group of six rats which had been digitalized for 3 days by the daily administration of 4 mg/kg of crystalline digitoxin in propylene glycol was given an additional injection of 5 mg/kg of digitoxin in propylene glycol intraperitoneally at the start of the glucose injection period. The controls received an equivalent amount of the solvent. Both groups were fasted for 24 hours. It can be seen from the data that there was a decreased amount of

liver glycogen in overdigitalized animals, the per cent of glycogen decreasing somewhat as the animals approached death. These results seem to indicate that increased glycogenesis occurred in the liver of rats given non-toxic doses of digitoxin, while toxic doses of the drug resulted in a decrease in the amount of liver glycogen in rats in which symptoms of over-digitalization are manifested.

*Summary.* There was no marked difference in the distribution of the acid-soluble phosphorylated intermediates of glycolysis in the heart muscle of normal and digitoxin-poisoned rats. Small differences in the glycogen values of heart values were noted, but there was an increased amount of liver glycogen in the digitalized animals as compared with the controls as indicated by an average of 2.39% of glycogen in the former and 1.85% in the liver of the latter. Neither was there any difference in the rate of breakdown of glycogen in the excised liver tissue in normal and digitoxin treated rats. Increased glycogenesis occurred in the liver of rats given non-toxic doses of digitoxin while toxic doses of the drug resulted in a decrease in the amount of liver glycogen in rats in which symptoms of over-digitalization were manifested.

The author wishes to express his appreciation to Dr. K. P. DuBois and to Dr. E. M. K. Geiling of the Department of Pharmacology for their interest and helpful advice in this problem.

## 16741

## Effect of Fibrinolysin upon Oxytocin, Vasopressin and Hypertensinogen.

H. CROXATTO, W. BADIA, AND R. CROXATTO.

*From the Laboratory of Physiology, Catholic University, Santiago, Chile.*

Plasma fibrinolysin has been considered a tryptase. Its proteolytic effect may be observed not only in the dissolution of fibrin but also in the digestion of fibrinogen, gelatin and casein, which is shown by the production of non-protein nitrogen when it acts upon these substrates (Tagnon *et al.*;[1] Kaplan *et al.*;[2] Christiensen,[3] Ferguson,[4] Shinowara,[5]). Nevertheless fibrinolysin does not inactivate hypertensin (Croxatto and Badia,[6]) a substrate which is attacked by all proteolytic enzymes tested up to now, including crystallized pancreatic trypsin (Croxatto[7]).

As fibrinolysin may appear in the bloodstream in its active form, an experimental study was carried out by observing its effect upon the hormones of the neurohypophysis-oxytocin and vasopressin- and upon hypertensinogen, in a similar way to that used before for trypsin. This also has the advantage of permitting a more detailed comparison between the effects of fibrinolysin and pancreatic trypsin. It has already been established that the latter is capable of inactivating hypertensin and vasopressin but not oxytocin (Croxatto[7]).

Hypertensinogen is attacked by trypsin but without the formation of hypertensin. Moreover after this enzyme has been used the substrate is incapable of producing hypertensin even when renin is added.

The results published in this paper show that fibrinolysin, just as trypsin, destroys the pressor effect of vasopressin but does not, under the same conditions, alter the effect of

1 Tagnon, H. J., Davidson, C. S., and Taylor, H. L., *J. Clin. Invest.*, 1942, **21**, 525.

2 Kaplan, M. H., Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1942, **21**, 533.

3 Christiensen, R. L., *J. Gen. Physiol.*, 1945, **28**, 363.

4 Ferguson, J. H., *Am. J. Med.*, 1947, **3**, 67.

5 Shinowara, J. G., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 456.

6 Croxatto, H., Badía, W., and Croxatto, R., *Bol. Soc. Biol.*, Santiago, 1948, **5**, 82.

7 Croxatto, H., *Revista de Medicina Aliment.*, 1943, **5**, 259.

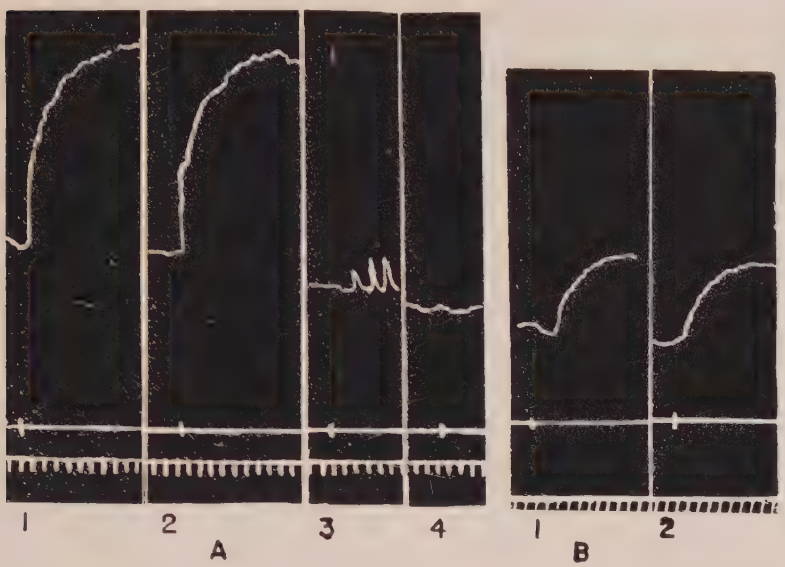



FIG. 1.

Contraction of the isolated guinea pig uterus. A, 1, 0.005 of 1.5 U Oxytocin; 2, 0.005 of 1.5 U Oxytocin + 5 mg fibrinolysin; 3, 0.005 of 1.5 U Oxytocin + 0.5 cc serum from patient in 9th month of pregnancy + 5 mg fibrinolysin; 4, 0.005 of 1.5 U Oxytocin + 0.5 cc pregnant woman serum. B, 1, 0.005 of 1.5 U Oxytocin + 20 mg fibrinolysin + 5 mg glutathione; 2, 0.005 of 1.5 U Oxytocin + 20 mg fibrinolysin. In A the incubation period lasted 24 hours, and 30 hours in B.

oxytocin. However hypertensinogen is not attacked by this enzyme, and after the addition of fibrinolysin it is still capable of producing hypertensin when renin is added.

*Material and method.* Three different samples of fibrinolysin were used, one prepared by us from ox plasma, activated with chloroform by the Loomis technic,[8] and two others obtained by fractionation of human plasma prepared by the Physical Chemical Department of Harvard Medical School.

The sample with which we did most of the experiments, (Squibb III-3-1) contained approximately 0.5 U per mg, according to Loomis. The substrates were: a) purified extract of neurohypophysis which contained 10 U.v. of oxytocin and 10 U of vasopressin per cc and b) human and horse hypertensinogen. In order to study the oxytocin fibrinolysin reaction a guinea pig uterus suspended in oxygenated Tyrode's solution was used. The blood pressure of the cat was employed in order to determine the inactivation of vasopressin by the enzyme. This same test was used to titrate the amount of hypertensin formed by the interaction of hypertensinogen with the enzymes:

*Effect of fibrinolysin upon oxytocin.* Different amounts of fibrinolysin: 2, 5 and 10 mg in 0.9% NaCl were made to act *in vitro* upon 2 U.v. of oxytocin; the solution was adjusted to pH 7.3 by means of a solution of sodium phosphate (0.2 normal). The total volume was brought up to 2.5 cc. The control contained 2 U.v. of oxytocin without the enzyme. The tubes were incubated at 37°C from 4 to 72 hours. When the incubation time exceeded 4 hours a drop of sodium merthiolate (Lilly) was added to each tube, as a bactericidal agent. As seen in Fig. 1 the oxytocic activity remains unchanged even after an incubation of 72 hours in the presence of 10 U of fibrinolysin, therefore it is quite clear that oxytocin is not hydrolyzed by fibrinolysin.

In experiments with pregnant woman serum it was proved fibrinolysin does not increase the destruction of oxytocin by the

[8] Loomis, C. E., George, C. H., and Ryder, A., *Arch. Bioch.*, **1947, 1, 12.**

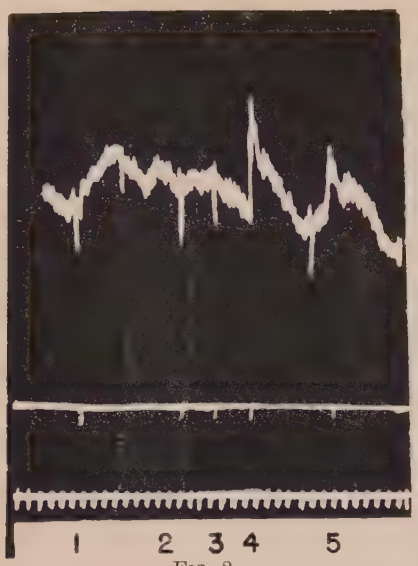



FIG. 2.

Arterial blood pressure of the cat. Injections into the femoral vein. 1, 0.3 of 1.5 U vasopressin + 0.1 mg trypsin; 2, 0.3 of 1.5 U vasopressin + 20 mg fibrinolyhin + 0.1 mg trypsin; 3, 0.3 of 1.5 U vasopressin + 20 mg fibrinolysin; 4, the same as in 3, but the different components were incubated separately. In all cases the incubation period lasted 24 hours at pH 7.3.

serum oxytocinase (Fig. 1).

*Effect of fibrinolysin upon vasopressin.* The incubation of vasopressin under similar conditions to those described above shows that fibrinolysin produces an appreciable inactivation of the pressor hormone. The titration tests were done by means of the blood pressure of the cat, anesthetized by dial. The solution was injected intravenously; in a few cases it was boiled previously. In order to discover whether fibrinolysin itself could have any influence upon the sensitivity of the cat to the pressor hormone, a mixture of fibrinolysin and vasopressin, incubated separately, was injected. Great care was taken to inject the same doses of substrate and enzyme as those which had been incubated.

As a general rule, each animal received only a few injections due to the tachyphylaxis produced by this hormone. In any case, by using a sufficiently large number of animals (3 or 4) it was clearly established that after 8 to 10 hours of incubation 10 to 20% of the vasopressin is inactivated when 10 U of fibrinolysin are used with 2 U of the pressor hormone. After 24 hours 40% is destroyed, and after 72 hours 80 to 100%. If crystallized trypsin is used in a dose equivalent to the fibrinolytic effect, it will be seen that 0.08 mg of this enzyme (containing 37% $MgSO_4$) which equals one fibrinolytic unit, destroys 0.8 U of vasopressin after 24 hours of incubation, *i. e.* the effect of 10 U of fibrinolysin, (Fig. 2).

The addition of 4 mg cysteine to 10 mg fibrinolysin, does not alter the effect of this enzyme upon vasopressin or oxytocin. This distinguishes it from blood plasma which shows a notable increase of its vasopressinase and oxytocinase effect when cysteine or glutathione are added (Croxatto, Reyes,[9]).

*Effect upon hypertensinogen.* Human and ox hypertensinogen were prepared as described, by precipitation with $(NH_4)_2SO_4$ at 0.5 saturation of the plasma, dialyzed with cold tap water and then filtered. The pH was brought to 7.4 by means of NaOH using a potentiometer. One hundred cc of this preparation incubated 15 minutes with 2 cc of the standard solution of pig renin gave 18 to 25 U of hypertensin. When hypertensinogen was treated with fibrinolysin for periods varying from 10 minutes to 12 hours, no pressor substance was produced. In order to investigate the possible formation of hypertensin, the mixtures of 50 cc of hypertensinogen with 1, 5, 10, and 20 mg of fibrinolysin were treated by the usual procedure for extracting hypertensin (Braun-Menéndez *et al.*[10]). The final extract was injected intravenously; the dose was calculated to be the equivalent of 10 to 20 cc of hypertensinogen. The results showed that the extract of hypertensinogen and fibrinolysin obtained produced exactly the same effect as the extract prepared from hypertensinogen alone, *i.e.* without hypertensin. In view of a previous paper (Croxatto and Croxatto,[11]) which demon-

[9] Croxatto, H., and Reyes, M., *Bol. Soc. Biol.*, Santiago, 1948, **5**, 80.

[10] Braun-Menéndez, E., Fasciolo, J. C., Leloir, L. F., and Muñoz, J. M., *J. Physiol.*, 1940, **133**, 731.

[11] Croxatto, H., and Croxatto, R., *Rev. argent. de Biol.*, 1948, in press.

activity were also noted at this time. Within a few hours the animal was lying on its side and severe dyspnea was observed. This condition became steadily worse, and no food or water was ingested. This state continued during the second and the third day, the animal becoming progressively weaker until it was unable to rise. Seventy-six hours after withdrawal, when the monkey was removed from its cage to be photographed, excitement and struggling led to further exhaustion and extreme weakness. Respiration was irregular, the animal was cyanotic, and it appeared to be moribund. Emergency measures such as intravenous glucose, injection of caffeine, etc., were attempted in an effort to save the animal, but they were without avail. Postmortem examination indicated an acutely dilated heart but no other significant changes were noted. The animal was generally in a good state of nutrition. Withdrawal signs in the other morphine animal were also severe, but not as striking as those just described.

Since these results are in essential agreement with those previously described by us,[1] it seems apparent that the normal monkey differs from the dog and from the former human morphine addict in response to this compound. The question must naturally be raised as to whether prolonged poisoning with morphine modifies or conditions the response of the human individual to subsequently administered methadon and/or other compounds and if so whether normal human subjects, previously not addicted to morphine react like the monkey or like the dog. In view of the fact that no primary case of methadon addiction has thus far been described in man, the evidence to date suggests that the response of the normal monkey is similar to that of the non-addict.

*Summary.* Racemic methadon administered thrice daily in maximal tolerated dose, does not induce a significant degree of physical dependence in the monkey (*Macaca mulatta*) confirming previous experiments involving single daily administration.

16744

## Activity Curves of Crude and Purified Inhibitors and Accelerators of Blood Coagulation.*

LEANDRO M. TOCANTINS AND ROBERT T. CARROLL.

*From the Division of Hematology, Department of Medicine, Jefferson Medical College and Hospital, Philadelphia.*

Most crude extracts of tissues possess in a variable proportion, clot inhibiting as well as clot accelerating substances.[1] Ordinarily, the clot accelerators are in dominance, and their action tends to mask the presence of the inhibitors. It seems from the evidence here presented that testing the activity of these extracts or their purified derivatives at different concentrations makes it possible to tell if they consist of such mixtures and, if so, whether they are *predominantly* clot accelerators or clot inhibitors. Moreover, the relative degree of purity (*i.e.* freedom from the antagonist) of the extracts is reflected by the character of their own activity curves. An extension of this concept has been made to the examination of the coagulant and anticoagulant content of other complex mixtures such as blood, plasma, and plasma fractions.

1. Activity of crude cephalin mixtures. Cephalin was prepared from acetone dried human brain by a method previously described.[2] *Crude cephalin* refers to the prod-

* Aided by a grant from the U. S. Public Health Service.

1 Tocantins, L. M., Carroll, R. T., and McBride, T. J., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 110.

2 Tocantins, L. M., *Am. J. Physiol.*, 1945, **143**, 67.

uct of only one precipitation of the ether-soluble lipid with cold absolute ethanol. This precipitate was washed once with acetone, the acetone removed and the waxy material well homogenized in a 1.4 g% solution in 0.85% NaCl, the pH being adjusted to 7.2 - 7.4. The activity of the preparation was then tested against citrated normal human plasma, collected and preserved with special precautions[3] and measured with collodion coated pipettes.[2] The clotting time of such plasma in collodion coated 13 mm wide tubes, at 38° (0.1 ml plasma, 0.1 ml 0.85% NaCl, 0.1 ml 0.02 M $CaCl_2$) ranges between 500 and 700 seconds.

Testing the activity of this crude cephalin at decreasing concentrations yields a curve with a biphasic course (Chart 1). The greatest clot accelerating effect was obtained at a concentration of the lipid extract in the plasma of 0.35 g%, which corresponds well with the optimum range of concentration (0.06 - 1.0%) for the activity of crude cephalin observed by Hanzlik and Weidenthal.[4] The crude cephalin at first gains clot accelerating power on dilution, then reverses itself and progressively becomes less potent. Such biphasic activity curves seem to be an expression of the coexistence of coagulants and their antagonists.

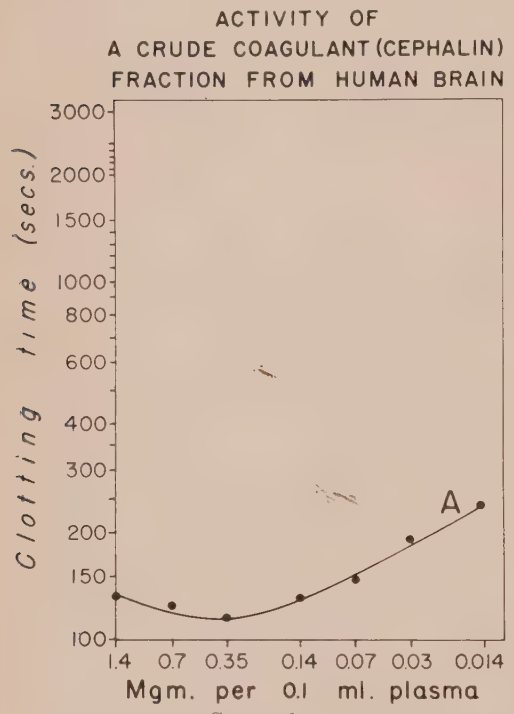



CHART 1.

Cephalin obtained from a single precipitation with absolute ethanol from an ether extract of human brain. In this and subsequent similar charts, the abscissa refers to total mg of the lipid coagulant or anticoagulant (or a mixture of both) added to 0.1 ml of recalcified citrated plasma.

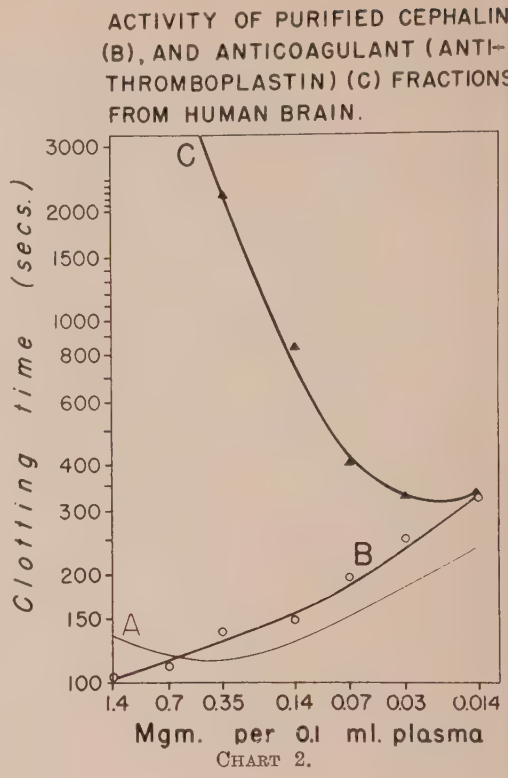



CHART 2.

2. Activity of purified extracts. The crude cephalin obtained by a single precipitation of the ether-soluble lipid was then purified by repeated precipitations with cold absolute ethanol. "Purified cephalin" is used to designate the ethanol insoluble lipid obtained after six successive precipitations. The mother liquors treated as described in method 1, elsewhere[1] yielded a clot-delaying lipid, here designated as "purified anticoagulant" (or antithromboplastin).

As shown in Chart 2, the purified coagulant

[3] Tocantins, L. M., *Am. J. Physiol.*, 1943, **139**, 265.

[4] Hanzlik, P. J., and Weidenthal, C. M., *J. Phar. and Exp. Ther.*, 1919, **14**, 157.

and anticoagulant fractions of the crude extracts have antagonistic actions, and moreover, their curves of activity (Curves B and C) no longer have the biphasic character of the crude preparation (Curve A, Chart 1). Freed of the inhibitor, the purified cephalin at 1.4 percent concentration is a more potent coagulant than the crude cephalin. The monophasic curves seem to represent the activity of preparations fairly free of their respective antagonists.

3. Activity of artificial coagulant-anticoagulant mixtures. These were prepared by mixing different proportions of solutions of the purified coagulant and anticoagulant fractions of the brain. The curves of activity for these mixtures (D,E,F, Chart 3) follow a course analogous to that of crude cephalin preparations. The mixtures with more coagulant (Curve F) exhibit the biphasic behavior at the higher concentrations, while those with more anticoagulant (Curve D) display it at lower concentrations.

The foregoing led us to test the activities of various fluids such as blood, plasma and cer-

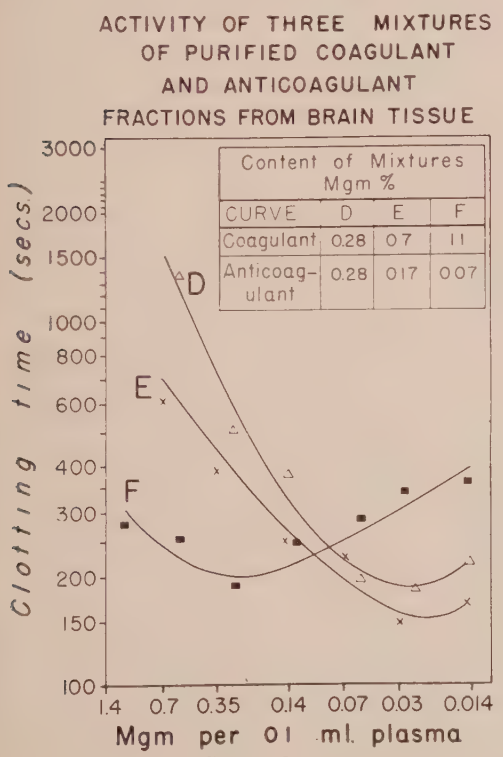


CHART 3.

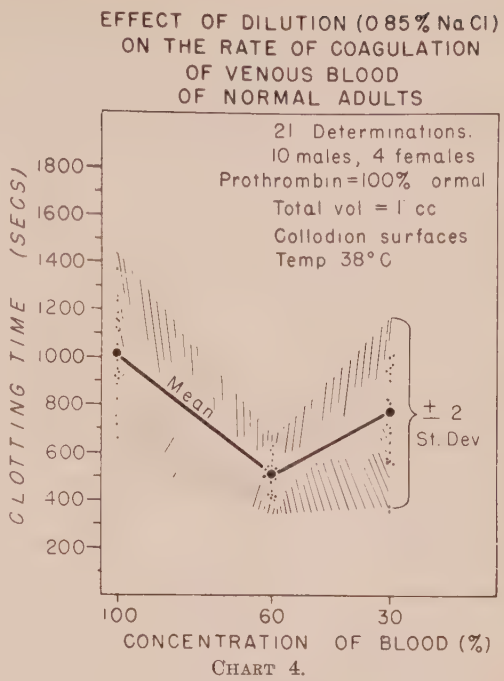


CHART 4.

tain plasma fractions separated by the Cohn method.[5]

Activity curves of blood and plasma. Blood was collected with special precautions[3] and placed into 3 collodion coated tubes. One ml was placed in the first tube; the amount of blood in each of the other 2 tubes was so adjusted that in the second tube the blood was diluted to 60% of its volume (0.6 ml blood, 0.4 ml 0.85% NaCl) and in the third tube to 30% (0.3 ml blood, 0.7 ml 0.85% NaCl). All three tubes were gently tilted 2 or 3 times and kept at 38°C. As shown in Chart 4, dilution of the blood to 60% of its volume accelerates its coagulation, and even when diluted to 30%, the blood still clots faster than when undiluted. Blood from hemophilics is even more strikingly affected by dilution (Chart 5).

Hypercoagulable blood, as observed after a severe hemorrhage, is little affected by contacting surfaces of different types[6] and clots nearly as rapidly in glass as in collodion tubes. Dilution of such blood prolongs its rate of coagulation from the start (Chart 6), an indi-

[5] Cohn, E. J., *Blood*, 1948, **3**, 471.

[6] Tocantins, L. M., *Blood*, 1946, **1**, 156.

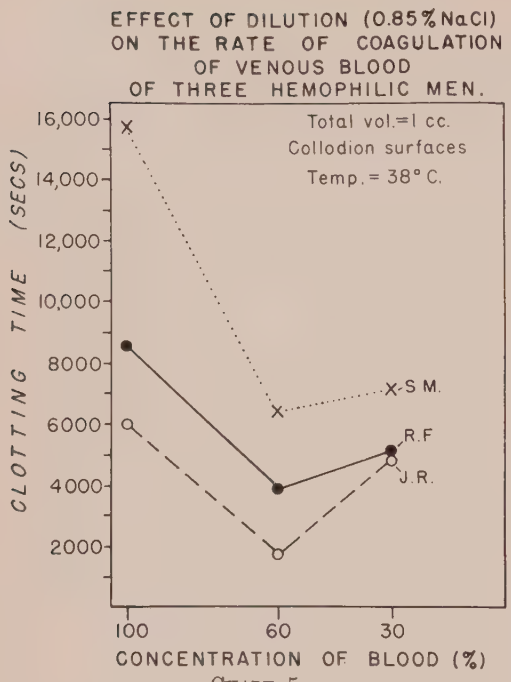



CHART 5.

Dilutions carried out as explained in text. Prothrombin concentration of undiluted blood (1-stage method) 100% of normal.

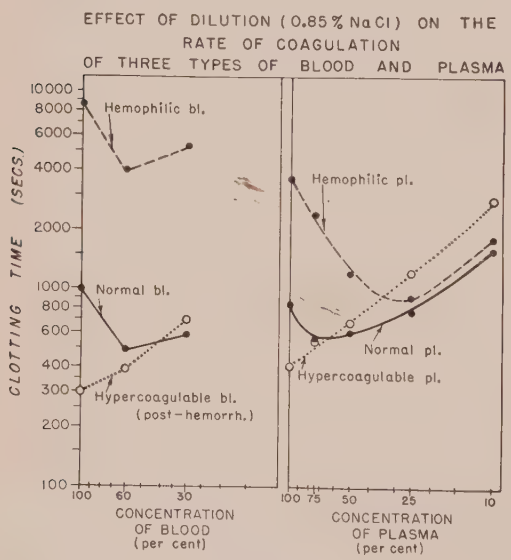



CHART 6.

Effect of dilution on the rate of coagulation of hemophilic, normal, and post-hemorrhagic blood and plasma. Blood diluted as described in text. Plasma clotting mixtures: 0.1 ml plasma, 0.1 ml 0.85% NaCl, 0.1 ml $CaCl_2$ (molar concentration adjusted to dilution of plasma); collodion coated tubes at 38°C.

cation that coagulants are predominant, the inhibitors having been reduced either by blood dilution *in vivo* during the hemorrhage, or offset by entrance of coagulants (thromboplastin) into the circulation.

The effect of dilution on hypocoagulable (hemophilic), normal and hypercoagulable (post hemorrhagic) plasmas is similar to that on whole blood (Chart 6). Hypercoagulable plasma which when undiluted clots many times faster than hemophilic plasma, actually clots slower than the latter, when both are diluted to below 20% of their original concentration. If the curves on Chart 6 are compared with those of the mixtures of purified coagulant and anticoagulant (Chart 3), it is apparent that the hemophilic plasma behaves like a predominantly anticoagulant mixture (Curve E, Chart 3) while the post hemorrhagic plasma is more like a predominantly coagulant solution (Curve B, Chart 2).

The clot-accelerating effect of dilution is best demonstrated in paraffin or collodion.[2] In glass the principal effect of dilution is to prolong the time,[7] for glass itself accelerates coagulation and dilution of the blood will only reduce that effect. Even in glass, however, the clotting time of *hemophilic* plasma is substantially reduced by dilution,[2] though the changes are not as impressive as in collodion or plastic tubes.

5. Activity Curves of Plasma Fractions. The dry plasma fractions were obtained from Dr. Cohn's laboratory and the biological laboratories of Sharp and Dohme. Three per cent solutions were made in 0.85% NaCl, and the pH adjusted to between 7.0 and 7.3. The fractions were tested on recalcified citrated normal human plasma, as described before.

With the exception of fraction V all fractions had clot-accelerating activity at concentrations between 0.7 and 3%. Fraction III was the most potent due perhaps to its prothrombin and thrombin content.[5] Fraction V was apparently inert; it did not seem to have either coagulant or anticoagulant action. The curves of activity for fractions IV-1 and IV-4,

[7] Copley, A. L., and Houlihan, R. B., *Science*, 1944, **100**, 505.

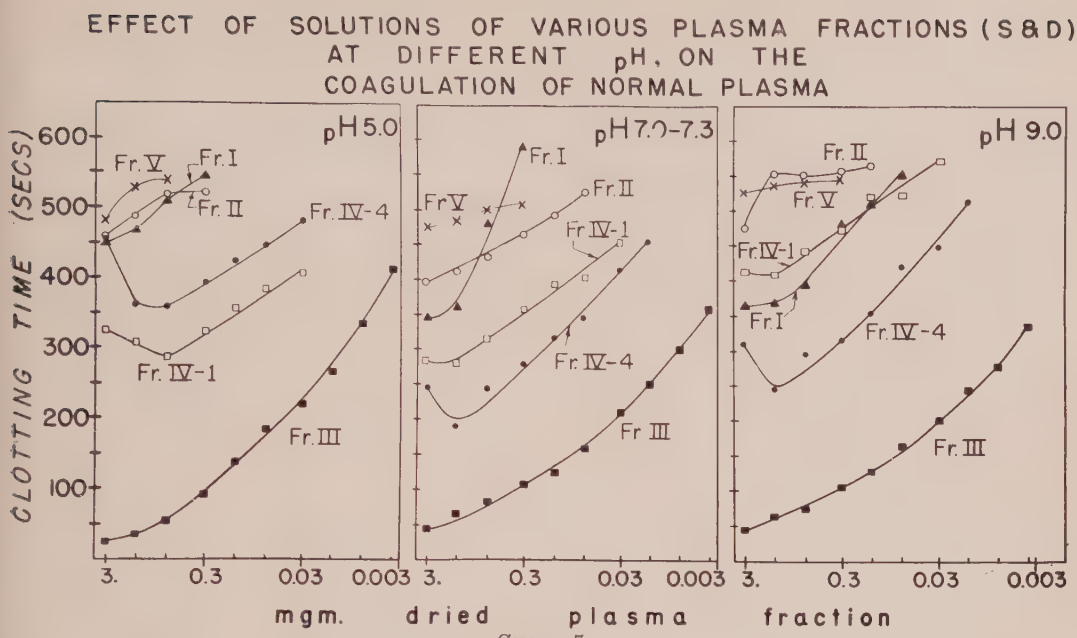



CHART 7.

The pH of the solutions was adjusted with dilute NaOH or HCl. A 3% solution of each fraction in $H_2O$ was prepared; dilutions were made with 0.85% NaCl. 0.1 ml fraction, 0.1 ml normal citrated plasma, 0.1 ml $CaCl_2$ (optimum molar concentration for each fraction/plasma mixture.) Fraction I usually contains the largest amount of citrate.

especially the latter, were of the biphasic type. Testing the activity of the fractions in an acid solution (pH 5.) seemed to accentuate the biphasic behavior of fractions IV-1 and IV-4. Even at pH 9, however, the trend of the curves was not significantly altered (Chart 7). The course of the curves suggested that these two fractions contained anticoagulant factors. A good portion of the lipoprotein complexes of the plasma is in fraction IV,[8] and it has also yielded the highest content of the lipid antithromboplastin.[9]

*Discussion.* These observations seem to make it desirable that the activity of purported clot-accelerating and inhibiting substances be tested over a wide range of concentrations, using stable plasma as a substrate, held in surfaces which do not themselves accelerate coagulation. Only in this manner may it be possible to detect both coagulant and anticoagulant activity, since excess of one may mask the action of the other, when the material is tested at a single concentration. The most striking example of this behavior is found in fraction IV-4 from which an active anticoagulant may be separated,[9] yet the intact fraction is a clot accelerator.

The slope of the activity curve of the purified anticoagulant is much steeper than that of the purified coagulant. Within the range of 0.35 - 0.07 mg the relation between concentration of the material and clotting time is linear (Chart 2). The anticoagulant gains (or loses) activity in this range, at a considerably more rapid rate than the coagulant. The activity curves of the artificial mixtures (Chart 3) compromise between the steep slope of the purified inhibitor and the gradual straight rise of the purified coagulant.

The biphasic course of the activity curves of normal blood and plasma seem to indicate that these fluids themselves are complex anticoagulant-coagulant mixtures. The fact that diluted blood, though containing less pro-

8 Mulford, D. J., *Ann. Rev. Physiol.*, 1947, **9**, 327.

9 Carroll, R. T., and Tocantins, L. M., Separation of a Lipid Antithromboplastin from Blood, Plasma and Plasma Fractions; presented at the International Congress of Hematology, Buffalo, August 23, 1948.

thrombin, platelets, ac-globulin and fibrinogen clots faster than intact blood, is an indication of the effectiveness of inhibitors in blocking or offsetting changes in these coagulation factors. Moreover, since the coagulation of hemophilic blood or plasma is greatly shortened by dilution, it is difficult to understand how a deficiency of a plasma constituent can be responsible for the defect in this disorder. Dilution would naturally tend to accentuate the deficiency and thereby further delay coagulation.

*Summary.* Crude and purified cephalin and antithromboplastin extracted from brain tissue yield characteristic activity curves when tested at different concentrations, on stable citrated normal human plasma. Moderate dilution accelerates the coagulation of normal and hemophilic blood and plasma and delays that of posthemorrhagic blood and plasma. Excepting fraction V, all Cohn's plasma fractions have coagulant action; only fractions IV-1 and IV-4 display a biphasic curve of activity, an indication that they are mixtures of coagulants and anticoagulants.

## 16745

## Does Administration of Diethylstilbestrol to Pregnant Women Result in Increased Output of Urinary Pregnanediol?*

M. EDWARD DAVIS AND NICHOLAS W. FUGO.

*From the Department of Obstetrics and Gynecology, and of Pharmacology, The University of Chicago and The Chicago Lying-in Hospital.*

In 1946 there appeared in the literature the interesting observation that the oral administration of diethylstilbestrol to a pregnant diabetic woman resulted in an increased excretion of urinary pregnanediol as measured by the Venning method.[1] This finding was interpreted by the authors as an index of increased production of steroids by the placenta brought about by estrogenic stimulation. They suggested the oral administration of diethylstilbestrol in the prevention and treatment of the accidents of late pregnancy such as the toxemias, premature fetal death and diabetic complications associated with gestation.

In an earlier report[2] we were unable to demonstrate increased pregnanediol excretion in patients receiving large amounts of diethylstilbestrol early in pregnancy. During the past 2 years we have studied a large group of patients who presented a variety of pregnancy complications in early and late gestation. Large amounts of diethylstilbestrol (5 to 200 mg daily) were administered to these women and bi- and tri-weekly urinary pregnanediol determinations were made by a method described by us in a previous communication.[3] No obvious increase in free pregnanediol was apparent in any of our studies. The results of these observations are in the process of publication.

The apparent discrepancy in the results obtained from the administration of diethylstilbestrol to pregnant women by the Smiths and in our own laboratory led us to seek an explanation. It has been reported in numerous publications[4–7] that in the metabolism of diethylstilbestrol it is conjugated and ultimately excreted as a glucuronide. The Venning

* This work has been done under a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

1 Smith, O. W., Smith, G. V. S., and Hurwitz, D., *Am. J. Obst. and Gynec.*, 1946, **51**, 411.

2 Davis, M. E., and Fugo, N. W., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 283.

3 Davis, M. E., and Fugo, N. W., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 39.

4 Zondek, B., and Sulman, F., *Nature*, 1939, **144**, 596.

5 Stroud, S. W., *J. Endocrinology*, 1939, **1**, 201.

6 Mazur, A., and Shorr, E., *J. Biol. Chem.*, 1942, **144**, 283.

TABLE I.
Simultaneous Urinary Pregnanediol Determinations by the Venning Method and the Free Pregnanediol Method in Normal Pregnant Patients and in Pregnant Patients Who Were Taking Diethylstilbestrol.

| No. | Free preg.,* mg/24 hr | Administered diethylstilbestrol, mg | NaPG* calculated as preg., mg/24 hr |
|---|---|---|---|
| 1 | 11.93 | 0 | 7.91 |
| 2 | 19.72 | 0 | 15.54 |
| 3 | 19.68 | 0 | 18.00 |
| 4 | 28.99 | 0 | 28.75 |
| 5 | 35.40 | 0 | 33.80 |
| 6 | 48.80 | 0 | 48.70 |
| 7 | 11.53 | 25 | 13.98 |
| 8 | 41.90 | 50 | 60.44 |
| 9 | 11.64 | 50 | 35.26 |
| 10 | 36.85 | 100 | 69.15 |
| 11 | 28.73 | 115 | 75.42 |
| 12 | 20.77 | 125 | 60.10 |
| 13 | 31.05 | 125 | 83.55 |
| 14 | 51.46 | 125 | 96.13 |

* These are uncorrected results. No attempt has been made to account for losses during extraction.

method for the determination of urinary pregnanediol measures the glucuronide titer of the urine. Thus, other glucuronides than pregnanediol are included in the results obtained. The method that has been in use in our laboratory determines the amount of free pregnanediol after acid hydrolysis.[3] The following experiments were carried out to compare the results obtained by these two methods in normal pregnant women who were receiving no medication and in those to whom diethyl stilbestrol in varying amounts was administered.

*Methods and results.* A series of 24 hour urine collections from normal women during various periods of gestation were analyzed simultaneously by the Venning method and by the method for free pregnanediol used in our laboratory. There was an extremely close correlation in the results obtained by the two procedures (Table I). The figures tabulated are uncorrected since it seemed to us that some loss of pregnanediol would occur in both methods.

Simultaneous pregnanediol determinations by both methods were made in a second group of pregnant patients to whom varying amounts (25 to 125 mg) of diethylstilbestrol were administered daily. The Venning method yielded substantially higher values than the method used in our laboratory. Furthermore, the increase obtained by the Venning method over that obtained by the free pregnanediol procedure was roughly in proportion to the amount of diethylstilbestrol administered to the patient.

A small group of patients in various stages of pregnancy under observation in the hospital was used for the following experiment. The urine was collected for several 24 hour periods and urinary pregnanediol levels were determined by both methods. The patients then received a single oral dose of 200 mg of diethylstilbestrol and urine collections continued. These, too, were analyzed for pregnanediol. The results of the determinations prior to the administration of diethylstilbestrol were comparable in both procedures. However, there was a marked increase in the amount obtained by the Venning method following the administration of the estrogen. (Fig. 1) Furthermore, in most cases this increase occurred during the 24 hour period following the ingestion of the diethylstilbestrol but in one patient there was a short delay so that the increased amount did not appear until

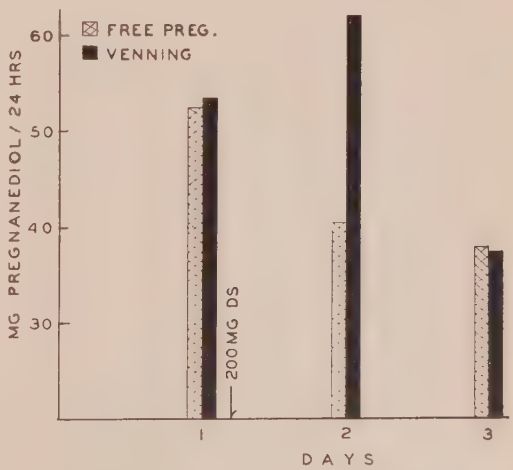



FIG. 1.
Simultaneous urinary pregnanediol determinations by the Venning method and the free pregnanediol method in a normal pregnant patient before and after the administration of diethylstilbestrol.

[7] Smith, A. E. W., and Williams, P. C., *Biochem. J.*, 1948, **42**, 253.

[8] Venning, E. H., *J. Biol. Chem.*, 1937, **119**, 473.

the second 24 hour urine collection. This patient had serious kidney damage which may have accounted for the delay in excretion. No attempt was made to determine the amount of diethylstilbestrol excreted in the feces.

Finally, the material obtained from the Venning procedures was assayed qualitatively for estrogenic activity using the castrated female guinea pig as the test animal. Opening of the vaginal canal by dissolution of the vaginal membrane was taken as the index of estrogenic activity. It was found that only those samples which showed an increased pregnanediol titer with the Venning method over the free pregnanediol method exhibited estrogenic potency during the period of observation.

*Summary and conclusions.* When diethylstilbestrol is administered to a woman during pregnancy, much of this material appears in the urine as a glucuronide. In the Venning method for the assay of urinary pregnanediol, all of the glucuronides are included in the final determination. Thus diethylstilbestrol glucuronide as well as pregnanediol glucuronide appears in the result obtained. The apparent rise in the urinary pregnanediol values obtained by the Venning method may be due to the ingested diethylstilbestrol which is conjugated and eliminated in the urine. The figures do not indicate an increased production of progesterone and resultant increased output of urinary pregnanediol.

Increasing amounts of diethylstilbestrol are being used in the treatment of pregnancy complications. In most instances the theory behind this therapeutic measure is that this estrogen stimulates steroid production. If urinary pregnanediol is to be used as a measure of increased steroid metabolism the value of diethylstilbestrol is open to question. It is possible that diethylstilbestrol exerts a favorable influence on placental circulation or on early placental development. However, there is no evidence that it results in an increased production of progesterone if urinary pregnanediol is to be regarded as an index of progesterone metabolism.

16746

## Age Factor in Estrogen-Induced Breast Cancers of Mice.*

MARTIN SILBERBERG AND RUTH SILBERBERG.

*From the Snodgras Laboratory, City Hospital, Saint Louis, Mo.*

In male mice of strains C3H and D, the age at the beginning of the treatment was one of the factors which determined the susceptibility of these animals to estrogen-induced breast cancers[1] and leukemias.[2-4] The breast cancer rate was higher in mice injected with estrogenic hormone before or about the onset of sexual maturity than in those receiving the hormone from the age of 4 to 6 months on. In female mice similarly treated, this age effect was much less conspicuous or lacking. According to Loeb,[1b] these findings suggest the following interpretation: (1) In younger mice, the breast tissue itself might be more susceptible to growth stimulation than in older animals. If this is the case, however, the lack of an age effect in females would be difficult to explain. Still, such an age factor might be present in female mice also, but it might be obscured or neutralized by the periodic stimulation of the mammary gland by the intrinsic estrogenic hormone produced in

* This investigation was supported by a research grant from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

1 (a) Loeb, L., *Harvey Lectures*, 1941, **36**, 228; (b) Loeb, L., *Biol. Symposia*, 1945, **11**, 197; (c) Loeb, L., Suntzeff, V., Burns, E. L., and Schenken, I. R., *Arch. Pathol.*, 1944, **38**, 52.

2 Murphy, J. B., *Cancer Res.*, 1944, **4**, 622.

3 Silberberg, M., and Silberberg, R., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 347.

4 Law, L. N., *J. Nat. Inst. Canc.*, 1947, **8**, 157.

the female during the estrus cycle. (2) The male sex hormone might have exerted an antagonistic effect on the carcinogenic action of the estrogenic hormone.[5-8]

The present experiments were carried out in order to obtain further information concerning the role of the age factor in estrogen-induced mammary cancers.

*Material and methods.* One hundred and twenty-three male mice of the closely inbred strain C3H, raised in our laboratory, and kept on a standard diet of Purina Laboratory Chow and water were used. Sixty-five animals were castrated at the age of 3 to 4 weeks. Twenty-three castrates (Group I) and 26 animals with intact testicles (Group II) received subcutaneous injections of 0.03 mg (200 Rat Units) of alpha estradiol benzoate† in sesame oil once a week for 5 months starting at the age of one month. The remaining 42 castrates (Group III) and 32 mice with intact testicles (Group IV) were injected with the same amount of hormone for the same length of time, but the treatment was begun at the age of 4 months. The animals were inspected at weekly intervals for the appearance of tumors and leukemia. Dead animals were examined for gross lesions. Tumor-bearing mice or animals which looked sick were killed. The tumor and 3 or 4 mammary glands, pieces of internal organs and some bones were removed and saved for histological studies. These findings will be reported at a later date.

*Observations.* The results are summarized in Table I. The first breast tumors were noted at the age of 7 months, and, therefore, mice living to this age and beyond are listed as animals "reaching the tumor age". These animals were distributed, as follows:

Group I: Castrates receiving the hormone from 4 weeks of age on: 18.

TABLE I.

| Experimental group | Total No. of animals | No. of animals reaching tumor age | Age at death of animals reaching tumor age Range (mo.) | Age at death of animals reaching tumor age Mean (mo.) | Animals with breast cancers No. | Animals with breast cancers % | Age at appearance of tumors Range (mo.) | Age at appearance of tumors Mean (mo.) |
|---|---|---|---|---|---|---|---|---|
| I. Male castrates receiving hormone at 4 wk | 23 | 18 | 7-18 | 12.9 | 8 | 44.4 | 7-15 | 9.7 |
| II. Males with intact testicles receiving hormone at 4 wk | 26 | 19 | 7-17 | 12.8 | 3 | 15.8 | 8-14 | 10.7 |
| III. Male castrates receiving hormone at 4 mo. | 42 | 40 | 7-19 | 13.0 | 12 | 30.0 | 8-19 | 12.8 |
| IV. Males with intact testicles receiving hormone at 4 mo. | 32 | 25 | 7-20 | 10.8 | 1 | 4.0 | — | 17.0 |

5 Lacassagne, A., and Raynaud, A., *Compt. Rend. Soc. Biol.*, 1939, **131**, 186.

6 Nathanson, I. T., and Andervont, H. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 421.

7 Gardner, W. U., *Cancer Res.*, 1946, **6**, 493.

8 Jones, E. E., *Cancer Res.*, 1941, **1**, 787.

† We are indebted to the Schering Corporation for the generous supply of Progynon-B.

Group II: Controls receiving the hormone from 4 weeks of age on: 19.

Group III: Castrates receiving the hormone from 4 months of age: 40.

Group IV: Controls receiving the hormone from 4 months of age on: 25.

In males with intact testicles injected with alpha estradiol benzoate for 5 months from the age of one month on (Group II), the cancer rate was 15.8%; the tumors appeared at a mean age of 10.7 months. In males with intact testicles treated similarly but receiving the hormone from the age of 4 months on (Group IV), the cancer incidence was 4%; the single tumor observed developed at the age of 17 months. Thus, in the younger control group, the estrogen-induced neoplasms were almost 4 times more numerous, and they appeared 6.3 months earlier than in the older control group. These results are essentially in agreement with the findings obtained by Loeb and his co-workers.[1c]

In males castrated at the age of 3 to 4 weeks and injected immediately with alpha estradiol benzoate for 5 months (Group I), the incidence of tumors was 44.4%; the neoplasms appeared at a mean age of 9.7 months. Orchidectomy thus raised the tumor rate in this age group about threefold and accelerated the appearance of the cancers by one month. Of males castrated at the age of 3 to 4 weeks but receiving the estrogenic hormone from the age of 4 months on (Group III), 30% developed mammary cancers as compared with 44.4% in the group injected at an earlier period of life, and the tumors were noted at a mean age of 12.8 months. Thus, in the older age group, castration increased the incidence of estrogen-induced breast cancers 7½ times and advanced the time of their appearance by about 4.2 months over that seen in mice with intact testicles.

*Discussion.* Orchidectomy performed before the onset of sexual maturity raised the incidence of estrogen-induced breast cancers. This increase was observed not only if the hormonal treatment was begun immediately after castration[9] but also, if the injections were started as late as 3 months after removal of the testicles. However, the results obtained in these 2 age groups differed in degree: Castrates injected from the age of one month on, showed a cancer rate about 50% higher than those in which the hormonal treatment was begun at the age of 4 months (44.4% in castrates of Group I as compared with 30% in castrates of Group III). These two groups of castrates thus showed variations in the incidence of estrogen-induced breast cancers similar to those occurring in the corresponding mice with intact testicles. However, whereas in the latter animals the tumor rate of the two age groups differed as much as 300%, the difference in injected castrates of the two age groups amounted to about 50%. This latter difference in the cancer rate of both age groups thus occurred in the absence of the testicles. It can, therefore, not be attributed to any inhibiting effect of the sex glands but it must be caused by certain extratesticular factors. It would be premature to define more precisely the nature of this age factor. It is presumably located in the mammary gland itself and constitutes a loss of responsiveness to estrogenic stimulation with advancing age, an interpretation which would be in agreement with the suggestion of Loeb.[1b]

A decrease of susceptibility may manifest itself not only in a lowered cancer incidence but also in a prolongation of the latent period of tumor development. In our present experiments, the latent period was apparently not influenced by the age of the castrates at the beginning of the hormonal treatment: In the younger castrates (Group I), the estrogen-induced breast cancers appeared 3.1 months earlier than in the older castrates (Group III), the mean tumor age being 9.7 months in the younger and 12.8 months in the older group. This difference in time corresponds to the 3 months' delay of the administration of the estradiol. This result may be correlated to the comparatively small difference in the tumor incidence of the two groups. On the other hand, in younger animals with intact testicles (Group II), estrogen-induced breast cancers appeared 6.3 months earlier

[9] Miller, E. W., and Pybus, F. C., *J. Pathol. and Bact.*, 1942, **54**, 155.

the action of diethyldithiocarbamate upon the oxygen uptake of active and blocked cells of the embryo of the grasshopper, *Melanoplus differentialis*. This compound is of especial interest since it has been extensively employed in methods dealing with the quantitative determination of copper. Loose physico-chemical compounds of copper are readily formed with it and this property, along with that of its being a carbamate, make it of especial interest for problems of cellular physiology.

*Material and methods.* The embryo of the grasshopper has proven to be extremely favorable biological material for investigating cellular reactions since it can be obtained both in a growing or mitotically active state as well as in a resting or blocked condition in which no mitotic activity is present.[4] Embryos were dissected from eggs in sterile phosphate buffered Ringer solution (pH 6.8) and treated as previously indicated.[5] All solutions of sodium diethyldithiocarbamate were made up in Ringer solution and final concentrations were calculated from the amounts added to the respiration flasks. Standard Warburg technics with flasks of 5 cc capacity were employed at 25°C. One hundred embryos were used in each flask and 12 to 18 manometers employed for each concentration of reagent.

*Results.* All remarks concerning the action of diethyldithiocarbamate should be prefaced with the fact that no significant differences in the reactions of mitotically active and blocked cells to this reagent have been found. Data presented, therefore, apply equally well to both physiological states of the cells.

The qualitative nature of the response of the oxygen intake of the embryonic cells to the reagent is strikingly similar for all concentrations employed. However, differences in degree of response, due to increased concentrations of the reagent, occur. Low concentrations produce only stimulation. In general (Fig. 1), an initial inhibitory period followed by a marked increase in oxygen consumption is noted for all high concentrations

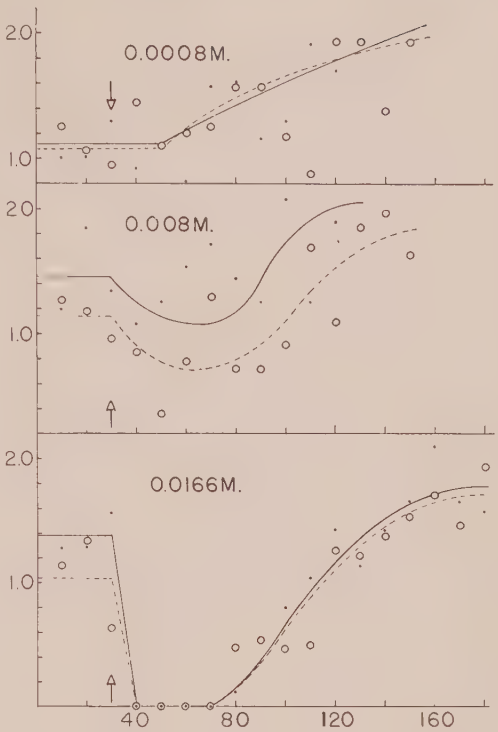



FIG. 1.

Shows effect of concentrations of diethyldithiocarbamate on the oxygen consumption of active and blocked cells of the embryo. Solid circles, active cells; open circles, blocked cells. Abscissa, time in minutes; ordinates, mm³ $O_2$ per 100 embryos per 10 minutes. Arrow indicates time of addition of drug. Concentrations in molarity indicated.

of the reagent. The length and degree of the inhibition and stimulation seem directly related to the concentration of the drug.

Embryos exposed to high concentrations for periods of 30 to 60 minutes and then washed and resuspended in Ringer solution, show almost complete recovery as indicated graphically in Fig. 2. As a matter of fact, the response of the embryo left exposed constantly to the reagent for periods comparable to those for washed embryos in Ringer solution, as in the above recovery experiments, show much higher rates of oxygen intake. Apparently constant exposure to the reagent is essential for the types of response normally shown by the embryos.

Since diethyldithiocarbamate is definitely known to unite with copper it becomes of some

[4] Slifer, E. H., *J. Morph. and Physiol.*, 1931, **51**, 613.

[5] Bodine, J. H., and Fitzgerald, L. R., *Physiol. Zool.*, 1948, **21**, 303.

interest to consider such a reaction as a possible way of throwing light upon the manner of its biological action. Copper acetate alone, when added to embryos produces a rather marked toxic effect (Fig. 3). Embryos exposed to the carbamate and then subjected to a similar concentration of copper acetate, show a rather marked decrease in the effects of the added copper. Such a result is probably due to the copper carbamate compounds formed which in turn are much less soluble and less toxic than the pure copper acetate. Whether the copper is added during the inhibitory or stimulating phase of the carbamate effect seems to make little difference in the results produced. This rather rapid and definite binding of copper by this reagent suggests that in its action upon the respiratory mechanism of embryonic cells some similar reaction may possibly occur. That sulfhydril-containing compounds have especial affinity for copper-bearing enzymes seems well established.[6]

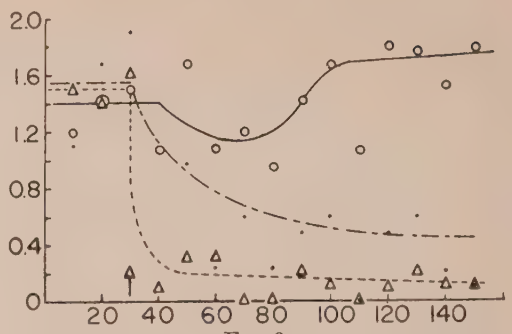



FIG. 3.

Shows the effect of additions of copper acetate and diethyldithiocarbamate upon the oxygen consumption of active cells of embryo. Abscissa, time in minutes; ordinate, mm³ $O_2$ per 100 embryos per 10 minutes. Arrow indicates time of addition of reagent from the sidearm into flask. Open circles, embryos in 0.0166 M diethyldithiocarbamate, Ringer solution added from sidearm. Triangles, embryos in Ringer solution, copper acetate added from sidearm (final conc. = 0.008 M). Solid circles, embryos in 0.0166 M diethyldithiocarbamate. 0.0252 M copper acetate added from sidearm (final conc. = 0.008 M).

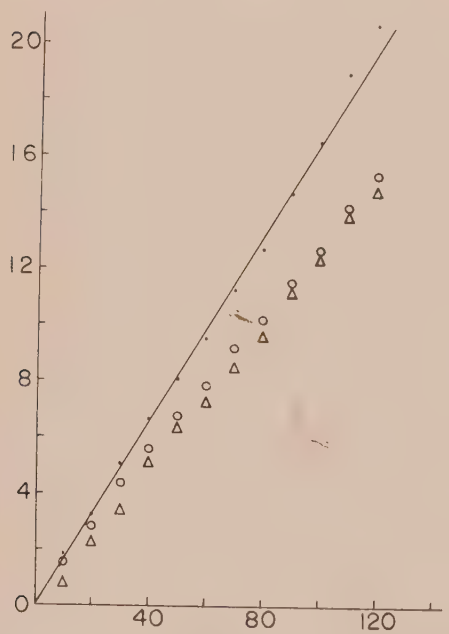



FIG. 2.

Shows recovery curves for embryos treated with diethyldithiocarbamate for 30- and 60-minute periods. Abscissa, time in minutes; ordinate, total oxygen (mm³) per 100 embryos. Solid circles, control embryos in Ringer solution. Open circles, similar embryos treated for 60 minutes in 0.0166 M diethyldithiocarbamate, removed, washed in Ringer and oxygen uptake determined. Triangles, embryos treated for 30 minutes with reagent and treated as for open circles.

A significant feature in the effects of diethyldithiocarbamate on the oxygen intake of embryonic cells is the striking difference in its responses compared with those for other carbamates such as ethyl carbamate (urethane). In the case of the latter, a stimulation occurs in low doses and a marked and constant inhibition in higher doses while for diethyldithiocarbamate low concentrations stimulate but with high concentrations an initial marked inhibition is invariably followed by stimulation. Further investigation seems necessary in order to definitely determine the reason for these basic differences in the reactions of such compounds.

*Summary and Conclusion.* 1. A study has been made on the effects of sodium diethyldithiocarbamate on the oxygen consumption of mitotically active and blocked embryonic cells of the embryo of the grasshopper, *Melanoplus differentialis*.

2. No significant differences in response of the active and blocked cells to the reagent are noted.

[6] Barron, E. S. G., and Singer, T. P., *J. Bio. Chem.*, 1945, **157**, 221.

3. In general, diethyldithiocarbamate produces stimulation in low doses and in high doses an initial inhibition followed by a marked stimulation in the oxygen intake of embryonic cells.

4. Partial recovery from high initial doses of the reagent occurs.

5. Copper salts are markedly antagonized when in the presence of the carbamate.

16749

## Alkaline Phosphatase of the Serum in Experimental Lathyrism of the White Rat.

ALFRED Y.-H. CHU,* ADAM A. CHRISTMAN, AND HOWARD B. LEWIS.

*From the Department of Biological Chemistry, Medical School, University of Michigan, Ann Arbor, Mich.*

Lathyrism, a condition associated with the consumption of considerable amounts of legumes of the genus *Lathyrus*, notably *Lathyrus sativus, clymenum*, and *cicera*, is a disease of frequent occurrence in man in certain regions of India, North Africa and Spain.[1-3] It has also been observed in domestic animals although marked species variation in susceptibility to the intoxicant has been noted. The causal agent, a toxic component of the seed, has not been isolated, nor is the mechanism of the production of the disease known. The intoxicant appears to act as a neurotoxin.[4]

Recently Rudra and Bhattacharya[5] have reported a marked increase in serum alkaline phosphatase content in human lathyrism. Ten male patients, ranging in age from 16 to 45 years were studied. Neither the duration nor the intensity of the disease was stated, although it was observed that "usually the serum-phosphatase level is a measure of the severity of the disease." In the group, the enzyme content of the serum ranged from 18.7 to 56.5 units (Bodansky), with an average of 38.7. In a group of 12 healthy males of the same age range, the phosphatase content varied from 0.8 to 5.7 units. The minimal value of the lathyrism group was 228% higher than the maximal value of the normal group.

Experimental lathyrism has been produced in young rats fed diets which contained considerable amounts of the seed of the flowering sweet pea (*Lathyrus odoratus*)[6,7] and other species of *Lathyrus*. In view of the marked increases in alkaline phosphatase of the serum reported in human lathyrism, we have studied the enzyme content of the serum in experimental lathyrism of the white rat.

In preliminary experiments with young (50 g) rats, difficulty was experienced in obtaining amounts of serum sufficient for serial determinations during the course of the experiments. Subsequently we have employed somewhat older animals in which it was possible to secure repeated samples of blood for analysis as the symptoms of lathyrism developed. With older animals, however, the onset of lathyrism is greatly delayed and the

* Fellow of the American Committee for Medical Aid to China. Present address: National Hsiang Ya Medical College, Changsha, Hunan, China.

1 Schuchardt, B., *Deut. Arch. klin. Med.*, 1886-87, **40**, 312.

2 Stockman, R., *J. Pharm. Exp. Therap.*, 1929, **37**, 43.

3 Díaz, C. J., *Revista clin. Español*, 1941, **3**, 303.

4 Filimonoff, I. N., *Z. ges. Neurol. Psychiatr.*, 1926, **105**, 76.

5 Rudra, M. N., and Bhattacharya, K. P., *Lancet*, 1946, **1**, 688.

6 Geiger, B. J., Steenbock, H., and Parsons, H. T., *J. Nutrition*, 1933, **6**, 427.

7 Lewis, H. B., Fajans, R. S., Esterer, M. B., Chen, C.-W., and Oliphant, M. J., *Nutrition*, 1948, **36**, 537.

TABLE I.
Alkaline Phosphatase of Serum of Rats Fed Sweet Pea and Edible White Pea Diets.

| Period on diet, days* | Diet | Phosphatase units (King-Armstrong) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Sweet pea | | | | | White pea | | | | |
| | Rat No. | 1 | 3 | 5 | 9 | 11 | 2 | 4 | 6 | 10 | 12 |
| 0 | | 85 | 89 | 83 | 67 | 59 | 142 | 82 | 101 | 70 | 62 |
| 8-17 | | 76 | 64 | 112 | 70 | 76 | 92 | 90 | 103 | 68 | 75 |
| 29-33 | | 80 | 67 | 115 | 70 | 66 | 80 | 129 | 136 | 81 | 109 |
| 63 | | 94 | 73 | 54 | 47 | 99 | 118 | 145 | 67 | 46 | 92 |

* The period at which samples of blood were collected varied somewhat within the ranges indicated for different rats. Rats 1 and 2, 3 and 4, etc., were paired in the feeding experiments.

changes in the bones[6,7] are less marked.

Ten adult male rats (200 g) were fed the diet described previously[7] (50% of ground sweet pea seed) for periods of 10 weeks. An equal number of controls, in paired feeding experiments, received the edible white pea of commerce (*Pisum sativum, var. arvense*). All animals which received the sweet pea diet developed lameness, spinal curvature, and, in a few cases, marked paralysis during the experimental period. Blood was obtained by clipping the end of the tail after the animal had been placed in a warm chamber at 56° for 5 min. After clotting, the serum was obtained by centrifugation; the centrifugate was decanted and the serum was centrifuged a second time. The upper portion of the second centrifugate was removed by a pipette and used for the phosphatase determination. Since the phosphatase content of white and red cells is much greater than that of the serum, it was necessary to remove all the cellular elements and to avoid hemolysis as much as possible. Alkaline phosphatase was determined by the method of King and Armstrong.[8] The results are reported as phosphatase units per 100 ml of serum (*i.e.* mg of phenol liberated by the hydrolysis of disodium monophenyl phosphate under standard conditions).

Results obtained with 5 pairs of animals are shown in Table I. They are typical of the values obtained with all 10 pairs. Although there was considerable variation in the phosphatase content both in the same rat over the experimental period and also in different animals, there was no evidence of any increase as the clinical symptoms of lathyrism developed, nor was there any significant difference between the values obtained with the control (white pea) and the experimental (sweet pea) groups. Certainly there was no indication of such a marked elevation of the alkaline phosphatase as has been reported by Rudra and Bhattacharya[5] in human lathyrism.

*Summary.* No significant changes in the concentration of alkaline phosphatase of the serum were observed in rats in which experimental lathyrism was produced by feeding diets containing 50% of sweet pea (*Lathyrus odoratus*) seed. This does not confirm the observations of Rudra and Bhattacharya in which the phosphatase values were markedly increased in human lathyrism.

We wish to express our indebtedness to the Ferry Morse Seed Company of Detroit for the sweet pea seed and to the Washburn-Wilson Seed Company of Moscow, Idaho, for the edible split pea seed.

8 King, E. L., and Armstrong, A. R., *Can. Med. Assn. J.*, 1934, **31**, 376.

Pyridoxamine acts like histamine, while aliphatic diamines activate rather than inactivate the tissue-ATP-ase and ophio-ATP-ase.

By these results histamine and ATP, which have been connected with the mechanism of shock production, are brought in a close relationship.

16753

## Failure to Produce Lesions or Auto-Antibodies in Rabbits by Injecting Tissue Extracts, Streptococci and Adjuvants.*

JOHN L. PECK† AND LEWIS THOMAS.

*From the Department of Pediatrics of the Johns Hopkins University School of Medicine, and the Harriet Lane Home for Invalid Children, Johns Hopkins Hospital, Baltimore, Md.*

Schwentker and Comploier[1] demonstrated the formation of complement fixing antibodies for homologous kidney tissue in rabbits which had been inoculated with a mixture of kidney tissue extract and staphylococcal or streptococcal toxin. Cavelti and Cavelti[2] subsequently reported that an antibody for kidney tissue could be demonstrated by the collodion particle-agglutination technic in rabbits and rats which were inoculated with a mixture of homologous tissue and Group A streptococci. The latter workers also reported that rats immunized in this fashion developed acute and chronic glomerulonephritis. Recently, Cavelti[3] described the development of antibody for homologous heart tissue in rats injected with heart extracts and streptococci, and also demonstrated the appearance of inflammatory myocardial lesions in these animals.

Kabat, Wolf and Bezer[4] and Morgan[5] have recently confirmed the earlier observation of Rivers and Schwentker[6] that the immunization of monkeys with extracts of homologous brain tissue leads to the formation of demyelinating lesions of the central nervous system which resemble those seen in multiple sclerosis. These lesions were brought about in greatly accelerated fashion by the use of the adjuvants described by Freund,[7] consisting of "Falba" or "Aquaphor", paraffin oil, and killed tubercle bacilli.

In the present study, the effect of inoculating rabbits with homologous heart and kidney tissue in combination with streptococci and Freund's adjuvants was studied.

*Material and methods. Rabbits.* Hybrid brown and grey male rabbits, weighing between 1.8 to 2.3 kg each, were used in all experiments.

*Tissue Suspensions.* Heart and kidney tissue were otained from normal rabbits. The organs were thoroughly perfused with physiological saline under sterile conditions before removal, and were either used immediately or stored whole in a $CO_2$ ice box. Antigens were prepared by grinding portions of each organ with abrasive, suspending in sufficient physiological saline to make a 10% suspension, and partially clearing by centrifugation for 5 minutes at 1500 R.P.M. Bacteriological cultures were made from samples of each suspension to ascertain sterility.

*Streptococci.* Several strains of beta hemo-

* This work was supported by a grant from the Life Insurance Medical Research Fund.

† Senior Pediatric Fellow of the National Research Council.

1 Schwentker, F. F., and Comploier, F. C., *Exp. Med.*, 1939, **70**, 223.

2 Cavelti, P. A., and Cavelti, E. S., *Arch. Path.*, 1945, **40**, 158.

3 Cavelti, P. A., *Arch. Path.*, 1947, **44**, 1.

4 Kabat, E. A., Wolf, A., and Bezer, A. E., *Exp. Med.*, 1947, **85**, 117.

5 Morgan, I. M., *J. Exp. Med.*, 1947, **85**, 131.

6 Rivers, T. M., and Schwentker, F. F., *J. Exp. Med.*, 1935, **61**, 689.

7 Freund, J., and McDermott, K., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 548.

lytic streptococci were employed: a group C strain (H46A) obtained through the courtesy of Dr. O. D. Ratnoff, and 3 Group A strains (type 19) recovered from the throat cultures of patients with acute tonsilitis (one of whom also had rheumatic fever). Eighteen-hour cultures of these organisms were grown in trypticase-soy broth, after which the streptococci were killed by heating at 56°C for 45 minutes.

*Adjuvants.* Heat-killed, dried tubercle bacilli, obtained through the courtesy of Dr. Jules Freund, were suspended in paraffin oil to a concentration of 0.4 mg per cc. Three parts of this suspension were transferred slowly, with constant stirring, to 2 parts of melted "Falba". The resultant mixture was immediately combined with other reagents as indicated below.

*Preparation of Antigen Mixtures.* Equal volumes of tissue suspension and streptococcus culture were mixed together, and 2 volumes of this mixture were added, drop by drop, to 5 parts of the adjuvant, with constant stirring. In control experiments, sterile broth replaced the streptococcus culture. Microscopic examination of each final suspension was made in order to ascertain that uniform globules of small size had been obtained.

*Inoculation of Animals.* Each rabbit was given 4 injections of 1 cc each, in separate subcutaneous areas. The injections were repeated at weekly intervals for a total of 3 weeks.

Each rabbit was bled from the ear before the first injection, and at 2-week intervals following the final injection, and the serum was stored in the frozen state. Sample animals were sacrificed in each experiment at 2, 4, 6, 8, and 10 weeks following the final injection of antigen, and portions of heart, kidney and other organs were placed in 10% formalin for subsequent histological examination.

*Complement Fixation Tests.* Tissue antigens for the complement fixation test consisted of 5 or 10% suspensions of heart or kidney tissue in physiological saline, which were made up on the day of testing and clarified by centrifugation at 2000 R.P.M. for 10 minutes. In some experiments, modified antigens were prepared by subjecting the tissue suspensions to a) centrifugation at 12,000 R.P.M. for 30 minutes, b) heating at various temperatures between 45°C and 80°C, and c) exposure to various pH levels between 4.5 and 9.5. The standard complement fixation test was employed, using 0.2 cc of complement (two units), 0.2 cc of the antigen, 0.2 cc of the serum under test, and, after a period of two hours at 37°C, 0.6 cc of sensitized sheep cells consisting of 0.2 cc of rabbit amboceptor (two units) and 0.4 cc of 2% cells. Fixation of complement was interpreted as occurring in those tubes which showed no hemolysis after 30 minutes in a 37°C water-bath.

*Collodion Particle Agglutination Test.* Collodion particles were suspended in normal rabbit heart and kidney extracts by the method described by Cavelti.[8] The agglutination of particles in varying dilutions of serum was tested by the method of the same author.

*Results.* Approximately 350 rabbits have been subjected to immunization with homologous tissue suspensions in combination with streptococci and Freund's adjuvants. Group C beta hemolytic streptococci were employed in the antigens used for one third of this group, and Group A streptococci in the remainder. In addition, a smaller number of control animals were injected with tissue suspensions alone, or with the suspensions plus adjuvants.

*Antibody Determinations.* As has been shown by Kidd and Friedewald,[9] normal rabbit serum contains a substance which causes fixation of complement with tissue suspensions from various rabbit organs. This "antibody" which is readily inactivated by heating at 65°C for 30 minutes, was encountered in most of the sera employed in this study, in titers of 1:16 or 1:32. In no instance, however, was a significant rise in complement fixation titer encountered between the pre-inoculation sera and the sera obtained at any period following

[8] Cavelti, P. A., *J. Immunol.*, 1944, **49**, 365.

[9] Kidd, J. G., and Friedewald, W. F., *J. Exp. Med.*, 1942, **76**, 543.

inoculation, with any of the antigens employed. Heating the sera at 65°C resulted in the disappearance of complement-fixing antibody from all sera tested. Treatment of the tissue antigens by high-speed centrifugation, heating, or exposure to different pH levels did not result in any positive complement fixation reactions.

The collodion particle technic yielded results which were uniformly negative in all experiments. No evidence of an antibody for heart or kidney tissue was demonstrable in the sera of rabbits following immunization with any of the materials employed.

*Pathological Studies.* No significant cardiac or renal lesions were demonstrable in any of the rabbits which were injected with tissue suspensions, adjuvants and Group C streptococci. The results obtained with Group A streptococci were also negative, with the exception of a single experiment involving twenty-one rabbits. In this experiment, 3 animals received kidney tissue suspension in combination with Group A streptococci and Freund's adjuvants, 3 received the tissue suspension with streptococci but without adjuvants, 3 received the tissue suspension with the adjuvants but without streptococci, and 3 received the adjuvants and streptococci without tissue suspension; a similar group of rabbits were given a suspension of heart tissue in the same combinations. The animals were sacrificed at the end of the sixth week after inoculation. All of the rabbits which received either heart or kidney tissue in combination with streptococci and adjuvants showed extensive areas of acute inflammatory reaction and muscle fiber destruction involving the right ventricle. These lesions did not resemble the characteristic lesion of rheumatic myocarditis. Similar but less extensive lesions were seen in 2 of the rabbits which were given the mixture of kidney suspension and streptococci without adjuvants, and in 2 of the rabbits which received heart suspension and streptococci without adjuvants. No lesions occurred in the hearts of the rabbits receiving tissue suspensions with adjuvants alone, or in the group receiving streptococci and adjuvants without tissue suspensions. No significant kidney lesions were seen in any of the experimental animals.

Many attempts were made to repeat these observations, employing the same strain of streptococcus and, so far as possible, the same materials and experimental conditions. All of these attempts were unsuccessful. No myocardial lesions which were in any way similar to those observed in the 10 rabbits referred to above were encountered again. Occasional small areas of infiltration by round cells were encountered between muscle fibers, but these seen with equal frequency in the heart tissue of normal untreated rabbits.

No explanation can be given for the myocardial inflammatory lesions in the single experiment described above. Although bacteriological cultures of the heart tissue and blood of these animals were negative, and the injection of serum from these rabbits into normal animals failed to produce any evidence of disease, it is considered possible that the myocardial lesions may have been due to an irrelevant infection of these animals by an unknown virus or bacterium. In view of the repeatedly negative results which were obtained when the experiment was duplicated, the significance of the myocardial lesions is doubtful.

*Summary.* The inoculation of rabbits with suspensions of homologous kidney or heart tissue, in combination with heat-killed cultures of Group A and Group C beta hemolytic streptococci and Freund's adjuvants, did not bring about the formation of detectable antibodies for homologous tissue.

Rabbits inoculated in this manner did not develop myocardial or renal lesions suggestive of rheumatic myocarditis or glomerulonephritis. In experiments involving approximately 350 rabbits, the only significant pathological finding consisted of acute inflammatory lesions in the myocardium of the right ventricle which occurred in ten animals. The latter animals had received injections of homologous heart and kidney tissue in combination with Group A streptococci and the adjuvants. The lesions did not resemble rheumatic myocarditis. Repeated attempts to confirm this observation, under similar conditions, yielded uniformly negative results.

16754

# Effect of Oxythiamine on Infection of Mice with the Lansing Strain of Poliomyelitis Virus.*

JAMES HAZLITT JONES, CLAIRE FOSTER, AND WERNER HENLE.

*From The Children's Hospital of Philadelphia and the Department of Biochemistry, School of Veterinary Medicine and the Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia, Pa.*

It was reported from this laboratory[1] that a dietary deficiency of thiamine increased the resistance of albino mice to the Lansing strain of poliomyelitis virus. A few months later Rasmussen, *et al.*[2] reported similar observations. Using Theiler's virus. Lichstein and associates in the same laboratory[3] found that deficiencies of some other dietary constituents afforded a similar degree of protection to mice.

However, none of the several other dietary deficiencies studied at The Children's Hospital of Philadelphia showed a degree of protection against the Lansing strain, which is considered a true poliomyelitis virus, equalling that of a deficiency of thiamine, although these included some of those reported by Lichstein as giving protection against Theiler's virus.

Because of the superior effect of a deficiency of thiamine in mice inoculated with the Lansing strain, a study of the influence of inhibitory analogues of thiamine on the resistance of mice to this virus was indicated.

The first thiamine analogue thoroughly studied in this laboratory was oxythiamine.† It is produced by replacing the $NH_2$ group on the pyrimidine ring of thiamine by an OH group. Some of the physiological properties of this analogue have been studied by Soodak and Cerecedo.[4] They found that mice receiving 1 mg of thiamine per day and given 25, 50 or 100 mg of oxythiamine daily, lost weight rapidly and died in about 2 weeks. They also showed that the enzyme of carp which destroys thiamine was inhibited by oxythiamine, but they did not determine the minimum amount of thiamine which would just off-set a given quantity of oxythiamine.

Several experiments were performed in the authors' laboratory studying the effect of this inhibitor of thiamine upon the resistance of mice to the virus of poliomyelitis. All of these gave the same general results. It is sufficient, therefore, to present in this paper the data obtained in the largest and most complete of the experiments.

*Experimental. Virus.* The virus and the technics employed were described in an earlier paper.[5] A recent test in this laboratory showed that the virus was still infectious for rhesus monkeys.

*Animals.* The Swiss white mice used in these studies came from the same colony that has been maintained in this laboratory for 14 years, and has produced mice for all of the resistance experiments conducted here.

The usual precautions described elsewhere[6] and essential for this type of experiment were

* Aided by a grant from the National Foundation for Infantile Paralysis.

1 Foster, C., Jones, J. H., Henle, W., and Dorfman, F., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 215.

2 Rasmussen, A. F., Jr., Waisman, H. A., Elvehjem, C. A., and Clark, P. F., *J. Bact.*, 1943, **45**, 85.

3 Lichstein, H. C., McCall, K. B., Kearney, E. B., Elvehjem, C. A., and Clark, P. F., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 279.

† We gratefully acknowledge the advice and cooperation of Dr. Gustav J. Martin of the National Drug Company of Philadelphia, Pa., who supplied the oxythiamine and provided the data on its structure, purity and microbiological assay.

4 Soodak, M., and Cerecedo, L. R., *J. Am. Chem. Soc.*, 1944, **66**, 1988.

5 Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *J. Exp. Med.*, 1944, **79**, 221.

6 Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *J. Exp. Med.*, 1944, **80**, 257.

taken. These included split litter technic for distribution, temperature and humidity control of the experimental rooms, and even a random distribution of the animals in the room to avoid the effect of any environmental differences. The mice were weighed daily, and following inoculation, each animal was removed from its cage for examination every 6 hours, day and night.

*Diet.* The basal complete diet had the following composition expressed in %: Casein, crude 25.0, cellulose, 2.0, salt mixture,[7] 4.0, linseed oil, 1.5, wheat germ oil, 1.0, glucose (cerelose) 66.5, carotene concentrate in oil, 5 drops, (Wyeth - 275 U.S.P. units pro-vitamin A per drop) Drisdol, 0.4 drop (Winthrop-250 U.S.P. units vitamin $D_2$ per drop). The B vitamins were supplied in the following quantities (mg per 100 g of diet.): Thiamine chloride 0.2, riboflavin 0.5, pyridoxine 0.5, calcium pantothenate 5.0, nicotinic acid 10.0, inositol 10.0, *p*-aminobenzoic acid 10.0, choline chloride 100.

This diet was given to the complete-diet control groups and to all of the oxythiamine groups. It was modified in the two low-thiamine groups only in so far as the thiamine content was concerned. Before the experimental regimens were initiated, all the animals were put on the complete diet for 2 days after they had been distributed into their individual experimental jars, in order to accustom them to the purified diet.

*Inhibition Index.*‡ Preparatory to the experiment, the inhibition index of oxythiamine was evaluated at 3 levels of thiamine: 3, 6, and 12 μg per mouse per day. In these titrations, both the thiamine and the oxythiamine were administered by mouth from a pipette. The inhibition index for the sample of oxythiamine used in the experiment reported here was found to be about 3 at all 3 levels of thiamine. Using *Lactobacillus fermentum,* the index in Dr. Martin's Laboratory was found to be 10, which is of the same general order as that obtained with the mice.

In the actual experiment this inhibition index was used as a guide, although the conditions were not quite comparable since the thiamine was given as an integral part of the diet, while the oxythiamine, on the other hand, was dissolved in saline and administered by pipette as above. In order to calculate, on the basis of the inhibition index, the amount of oxythiamine necessary to establish and maintain a given state of deficiency, the following assumptions had to be made. If the food intake for a normal mouse is 3 g per day, then each animal would receive 6 μg of thiamine. As the deficiency state advances under the influence of oxythiamine, the accompanying anorexia leads to a lower intake of diet and correspondingly a lower intake of thiamine. The oxythiamine dosage then has to be decreased by an estimated amount. It is worth commenting that with the pure samples of the analogue, severe signs of deficiency can be quickly terminated by reducing the oxythiamine dosage or by adding a suitable amount of thiamine.

*Experimental Groups.* The experimental animals were divided into 12 groups as presented in Table I. The mice of 6 of the groups were inoculated with a suspension of mouse brain infected with the Lansing strain of poliomyelitis virus (V), and for each of these there was a control group, the animals of which were injected with a suspension of uninfected ("normal") mouse brain (N).

The first pair of groups (1-V and 1-N) were fed the complete basal diet; the next 4 pairs of groups (2-V and 2-N, 3-V and 3-N, 4-V and 4-N, 5-V and 5-N) received the same complete basal diet and in addition were given oxythiamine; the last pair of groups (6-V and 6-N) were maintained on a low-thiamine diet, in which the intake of thiamine was varied between 20 and 60 μg per 100 g of diet. The amount of thiamine was regulated in an effort to maintain a level of deficiency that

[7] Jones, J. H., and Foster, C., *J. Nutr.*, 1942, **24**, 245.

‡ The inhibition index is defined as the quotient of the molecular amount of analogue divided by that molecular amount of metabolite which just counteracts the analogue. For example, if the inhibitory action of 1.0 millimole of analogue were just offset by 0.001 millimole of metabolite, then the index would be 1.0/0.001 or 1000 (see D. W. Woolley, *Advances in Enzymology,* 1946, **6**, 129.)

TABLE I.

| Group No. | No. of mice | Thiamine, mg per 100 g diet | Oxythiamine | Time of beginning oxythiamine in respect to day of inoculation | No. of mice in N-groups dying during experiment |
|---|---|---|---|---|---|
| 1-V* | 56 | .2 | — | None | — |
| 1-N† | 6 | .2 | — | " | 0 |
| 2-V | 57 | .2 | + | 9 days before | — |
| 2-N | 21 | .2 | + | 9 " " | 2 |
| 3-V | 57 | .2 | + | 2 " " | — |
| 3-N | 21 | .2 | + | 2 " " | 1 |
| 4-V | 60 | .2 | + | day of inoculation | — |
| 4-N | 21 | .2 | + | " " " | 1 |
| 5-V | 60 | .2 | + | 4 days after | — |
| 5-N | 22 | .2 | + | 4 " " | 1 |
| 6-V | 58 | .02-.06 | — | None | — |
| 6-N | 21 | .02-.06 | — | " | 0 |

* Groups with "V" in number received virus-infected brain.
† " " "N" " " " uninfected brain.

would keep the average weight more or less constant.

The 4 pairs of groups that received the oxythiamine differed among themselves in respect to the day the administration of the analogue was begun. An attempt was made with the first pair of these groups (Group 2-V and 2-N) to produce the deficiency gradually, and to time it so that the animals would be in the same state of deficiency on the day of inoculation as the mice on the diet low in thiamine. To that end 32 $\mu$g of oxythiamine, or about twice the amount that would be counteracted by the thiamine in the diet, were given per animal per day, starting 9 days before inoculation. As that was insufficient to produce a deficiency state in the desired time, the oxythiamine was increased to 64 $\mu$g 3 days later, but after 5 days on this amount it was necessary to reduce it to 8 $\mu$g and then to 4 $\mu$g in order to stop the rapid loss of weight of the animals. After 2 days on 4 $\mu$g and 2 additional days on 8 $\mu$g, their weights could then be held about stationary on 16 $\mu$g per mouse per day.

Groups 3-V, 4-V, and 5-V and their controls were given oxythiamine starting respectively 2 days before inoculation, on the day of inoculation, and 4 days after inoculation. In each of these cases, the dose of oxythiamine was 128 $\mu$g per mouse for the first and second days. This large dose produced an almost immediate fall in weight. From the third day on the dose was gradually reduced, and in each case finally levelled off at 16 $\mu$g per day.

The experimental animals were observed until the 28th day after inoculation when the experiment was terminated.

*Results.* Table I shows that there were very few deaths among the groups of mice receiving the "normal" brain. This indicates that the "deficiencies" were not responsible for many deaths in the groups inoculated with virus. This assumption is substantiated by the fact that paralysis or some other sign of poliomyelitis was seen in nearly all animals of the infected groups that died. The following discussion of data is concerned, therefore, only with the animals that were given virus.

The results are presented in Chart I. The curves show the daily cumulative deaths in per cent as ordinates plotted against days after inoculation, as abscissae. It can be seen that, except for a few days at the beginning of the experiment and again at the end, the curve for the animals on the complete diet was above that for any of the "deficient" groups. The curves for Groups 2-V, 3-V and 4-V are very much alike. In these groups, the maximum difference in percentage deaths between Group 1-V and each of the "deficient" groups came at about the 14th day after inoculation. At this point deaths in the "deficient" groups were roughly 50% of those in the group on the complete diet. The death rate in Group 5-V (animals given initial dose of oxythiamine 4 days after inoculation) was nearly equal to

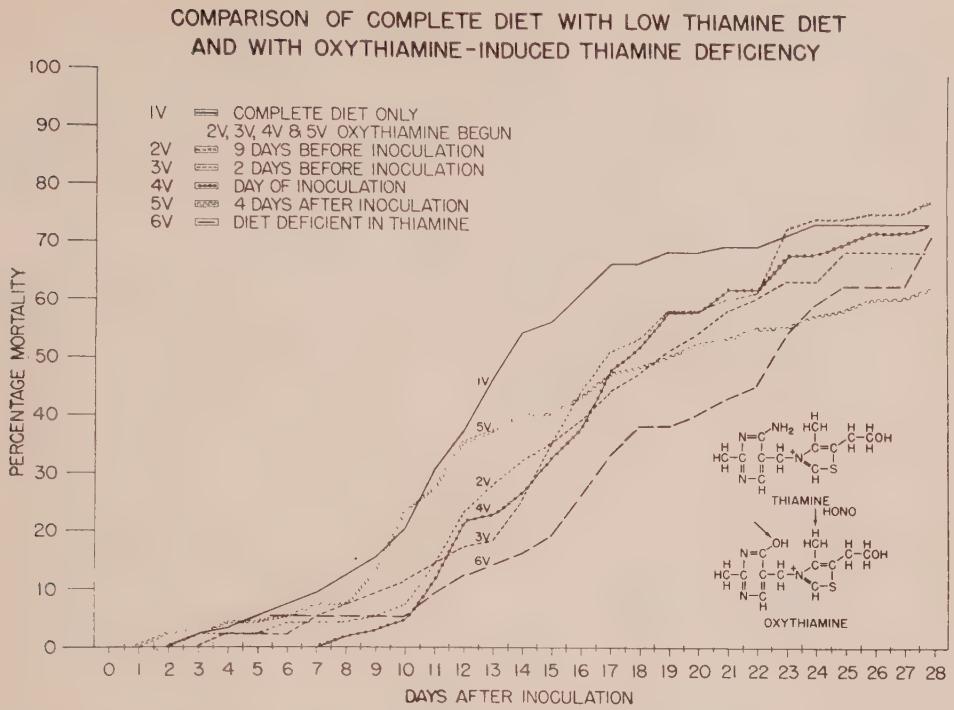



CHART I.

that of Group 1-V for the first 12 days. From then until the end of the experiment the death rate in Group 5-V was considerably decreased, so that even when the experiment was terminated, the percentage deaths was below those of the controls (63 to 74).

None of the groups of mice receiving the oxythiamine were protected to the extent of the animals on the low-thiamine diet. The cumulative percentages of deaths of each virus-inoculated test group were compared by the Chi-square method with the corresponding values for the animals of the virus-inoculated control group for each day of the experiment. The days after inoculation (inclusive) on which the differences were significant are as follows: Group 2-V, 10 to 15; Group 3-V, 12 to 18; Group 4-V, 6 to 17; Group 5-V, 17 to 20; and Group 6-V, 10 to 23.

*Discussion.* It seems to be possible to induce in mice a state of comparative resistance to poliomyelitis by means of an agent that can be given by mouth, the dose of which can be regulated within certain fairly narrow limits, and the deficiency signs quickly obliterated by suitable doses of thiamine. The protection resulting from oxythiamine treatment appeared to be of the same general order as that resulting from a diet deficient in thiamine, and presumably was associated with the presence of this induced deficiency. As in other deficiency studies, the protective action manifested itself mainly by a slowing of the death rate—a delaying rather than an outright protective action. However, as the animals of group 5-V were given some protection to the end of the experiment, it suggests that if the proper conditions could be found, it is possible that the degree of protection might be increased. It is of particular interest that in this same group (5-V), protection was afforded even though oxythiamine was not administered until 4 days after inoculation with the virus.

The difference between the greater protective effect of thiamine deficiency resulting from a low-thiamine diet, in contrast to the lesser protection afforded by the thiamine deficiency induced by oxythiamine, is not entirely explicable. One of the factors may have

been the difference in the weight of the mice in the two groups. The animals of oxythiamine groups were not given the analogue until a varying number of days after the low-thiamine group had been started on the diet deficient in the vitamin. During this interval, the mice which later received oxythiamine were growing at a normal rate, while the vitamin-deficient animals were growing subnormally. The growth-rate differential resulted in a difference of about 3 g between the thiamine-deficient and the oxythiamine-fed animals at the time of inoculation, which may indicate a difference in the degree of deficiency not otherwise recognizable.

*Summary.* Mice from a genetically controlled colony and under controlled environmental conditions were used to examine the effect of oxythiamine, an inhibitory analogue of thiamine, on resistance against the Lansing strain of poliomyelitis.

A significant degree of protection was induced in all the groups on the oxythiamine as compared with those on the normal diet. This protection was not quite as marked as in the mice on the low-thiamine diet.

16755

## Comparison of Penicillin G and A Biosynthetic Penicillin with Regard to Diffusion into Cerebrospinal Fluid.

WILLIAM P. BOGER AND WILLIAM W. WILSON. (Introduced by L. E. Arnow.)

*From Philadelphia General Hospital, Philadelphia, Pa.*

Under natural conditions *Penicillium notatum* or *Penicillium* chrysogenum produces a mixture of penicillins G, F, dihydro-F, K and X. The predominant form of the penicillin elaborated can be influenced by the addition of precursors to the nutritive media. Similarly, the addition of precursors that do not occur in nature has resulted in the formation of penicillins that do not occur naturally. Such penicillins are formed by reason of the ability of the mold to utilize these precursors and incorporate portions of them into the penicillin molecule.[1] Penicillins produced by the addition of precursors not occurring in nature may be referred to as biosynthetic penicillins. BT penicillin* is such a biosynthetic penicillin and will predominate if the precursor used is one containing the n-butylthiomethyl grouping in available form.

The widespread use of crystalline penicillin G (benzyl penicillin) has established the fact that many patients show the usual phenomena of drug sensitivity. In addition to the possible advantage of having a penicillin in which a substitution had been made for the benzyl group, it was thought that perhaps such a penicillin would have distinctive properties not possessed by benzyl penicillin. Preliminary observations made in animals suggested that BT penicillin might diffuse into the cerebrospinal fluid more readily than benzyl penicillin.[2] The investigation here reported was carried out to test this hypothesis.

*Materials used.* The water-soluble potassium salt of BT penicillin with a potency of 2900 units per mg was employed throughout. The material contained a small amount of penicillin G as a contaminant, for the media upon which the mold was grown during the preparation of this material contained naturally occurring precursors which permitted the formation of penicillin G. The sodium salt of crystalline penicillin G was used for comparison with the BT penicillin.

*Patients studied.* Eleven patients with central nervous system syphilis were investigated.

[1] Biosynthesis of Penicillins, *Science*, 1947, **106**, 503.

* n-butylthiomethyl penicillin supplied through the courtesy of Dr. N. P. Sullivan of Eli Lilly & Co.

[2] Sullivan, N. P., personal communication.

TABLE I.
Plasma Concentrations of Penicillin After Initial Intravenous Injection of 500,000 Units.

| Patient | Age, yrs | Color | Sex | Penicillin G* Min. after injection 5 | Penicillin G* Min. after injection 120 | Penicillin BT† Min. after injection 5 | Penicillin BT† Min. after injection 30 | Penicillin BT† Min. after injection 120 |
|---|---|---|---|---|---|---|---|---|
| BC | 57 | B | F | 63.49‡ | .992 | 34.56 | 4.32 | .270 |
| MW | 35 | W | F | 46.08 | .720 | 23.04 | 2.16 | .180 |
| RR | 35 | B | M | 67.58 | .248 | 34.56 | 5.74 | .270 |
| JS | 43 | B | M | 70.92 | 1.08 | 23.04 | 2.88 | .180 |
| JO | 52 | W | M | 46.08 | .744 | 34.56 | 2.16 | .360 |
| McL | 38 | W | M | 31.74 | .496 | 17.18 | 2.16 | .180 |
| IJ | 35 | B | M | 34.56 | —§ | 17.18 | 2.16 | .180 |
| JM | 44 | W | M | 23.81 | .996 | 34.56 | 2.88 | .360 |
| HZ | 63 | W | M | 63.49 | 1.98 | 39.94 | 3.74 | .360 |
| MK | 41 | W | F | 31.74 | 1.49 | 34.56 | 6.66 | .270 |
| WW | 61 | W | M | 63.49 | 1.49 | 64.51 | 13.31 | 1.66 |
| | | | Avg | 49.36 | 1.02 | 32.51 | 4.37 | .388 |

* Sodium penicillin G in aqueous solution.
† n-butylthiomethyl penicillin in aqueous solution.
‡ Oxford units of penicillin per cc of plasma.
§ Specimen broken.

There was no impairment of general health and no evidence of inflammation of the meninges.

*Method of study.* Each patient was observed during both periods of the study and each served as his own control. Following a single intravenous injection of 500,000 units of crystalline penicillin G (injection time 5-15 seconds), blood specimens were obtained at 5 and 120 minutes and a specimen of cerebrospinal fluid was obtained from the lumbar subarachnoid space at 120 minutes. These specimens were submitted for penicillin assay by a modification of the Rammelkamp serial dilution method. After an interval of no less than 3 days and no more than 7 days the patient received a single intravenous injection of 500,000 units of BT penicillin and thereafter blood specimens were obtained at 5, 30 and 120 minutes and a cerebrospinal fluid specimen was obtained at the end of 120 minutes. These specimens were likewise assayed for their penicillin content.

*Results.* The results are presented in Tables I and II. In Table I it may be observed that the average penicillin plasma concentrations resulting from the intravenous injection of 500,000 units of penicillin G were 49.36 units per cc at 5 minutes and 1.02 units per cc at 120 minutes. Following the administration of 500,000 units of BT penicillin, plasma concentrations were 32.51 units per cc at 5 minutes and 0.388 unit per cc at 120 minutes. The difference between the plasma concentrations observed at 5 minutes following the use of the two penicillins is not significant since the difference represents only a one tube difference in the Rammelkamp serial dilution method.[3] The difference between 1.02 units per cc and 0.388 unit per cc observed at the end of 120 minutes represents a difference of 4 tubes in the Rammelkamp assay and is therefore significant, but the difference is not great enough to postulate any essential difference in rate of elimination of the two penicillins. In Table II it may be observed that, at 120 minutes after the injection of penicillin G, 8 of 11 patients showed assayable quantities of penicillin in the cerebrospinal fluid whereas only 4 of 11 patients showed penicillin in the cerebrospinal fluid following the injection of BT penicillin. These results are barely significant statistically ($p = 0.057$). The difference in actual penicillin concentration observed, that between 0.032 following penicillin G and 0.01 following BT penicillin, is statistically significant ($p = 0.02–0.05$).

*Summary.* BT penicillin (n-butylthiomethyl penicillin) gave no evidences of toxicity, either immediate or delayed, following the

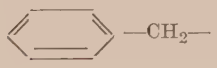
[3] Miller, A. K., and Boger, W. P., *Am. J. Clin. Path.*, 1948, **18**, 421.

TABLE II.
Penicillin in Cerebrospinal Fluid Two Hours After Initial Intravenous Injection of 500,000 Units.

| Patient | Age, yrs | Color | Sex | Penicillin G* | Penicillin BT† |
|---|---|---|---|---|---|
| BC | 57 | B | F | .045 | .0 |
| MW | 35 | W | F | .0 | .0 |
| RR | 35 | B | M | .031 | .0 |
| JS | 43 | B | M | .045 | .0 |
| JO | 52 | W | M | .0 | .045 |
| McL | 38 | W | M | .0 | .0 |
| IJ | 35 | B | M | .023 | .022 |
| JM | 44 | W | M | .031 | .0 |
| HZ | 63 | W | M | .045 | .0 |
| MK | 41 | W | F | .090 | .022 |
| NW | 61 | W | M | .045 | .022 |
| | | | Avg | .032 | .010 |

* Sodium penicillin G in aqueous solution.
† n-butylthiomethyl penicillin in aqueous solution.
‡ Oxford units of penicillin per cc of cerebrospinal fluid.
§ Any penicillin concentration less than 0.022 unit per cc has been regarded as zero.

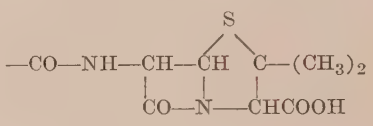
Formula of R



Benzyl or G

$CH_3—CH_2—CH_2—CH_2—S—CH_2—$
n-Butylthiomethyl or BT

Penicillin Nucleus



Structural comparison of the two penicillins employed in this study.

intravenous injection of 500,000 units in 11 patients. From the work here reported it appears that BT penicillin does not diffuse into the cerebrospinal fluid as readily as benzyl penicillin and plasma concentrations resulting from its use are essentially the same as those following the use of benzyl penicillin.

The authors wish to express their indebtedness to Doctors Harvey Bartle, Robert Bookhammer, Raphael Durante, Herbert Freed, and Anthony Frignito for their kindness in allowing us to study patients on their psychiatric services at the Philadelphia General Hospital; to Miss Barbara V. Prey for penicillin assays here reported, and to Mr. Joseph L. Ciminera for the statistical analysis of data.

16756

## Effect of Necrosin on Spontaneous Tumors in Mice.

VALY MENKIN.

*From the Agnes Barr Chase Foundation for Cancer Research, Temple University School of Medicine, Philadelphia, Penn.**

The pattern of injury with inflammation has recently been found by the writer to be referable to the liberation of a toxic substance by injured cells.[1] This substance is located in or at least it is associated with the euglobulin fraction of exudates of dogs and of man. It has been termed necrosin. Recent as yet

* Aided (in part) by a grant from the National Advisory Cancer Council.

[1] Menkin, Valy, *Arch. Path.*, 1943, **36**, 269.

unpublished studies have demonstrated a similar substance in injured tissues of invertebrates. The presence of necrosin in canine exudates has been confirmed by Smith and Smith[2] and by Tanturi.[3] Ludford has shown that trypan blue tends to accumulate in some of the cells of the stroma of tumors.[4] Duran-Reynals has demonstrated that the dye T-1824 and other poorly diffusable dyes localize from the circulating blood in spontaneous tumors of mice, rabbits, and of chickens.[5] He also showed that the sera of various animals localize from the blood into transplantable and spontaneous tumors of mice.[5] Duran-Reynals interpreted his findings in the light of a greater permeability on the part of the capillaries of a tumor than under normal circumstances.[5]

In view of these findings it was thought it would be of interest to see whether necrosin, being associated with a protein fraction, would also localize, when injected subcutaneously and at a distance from the tumor, into the tumor substance itself. The effect of the localization of such a toxic substance into experimental tumors would doubtless also yield valuable information. The experiments to be presently reported represent the first of a series of such observations.

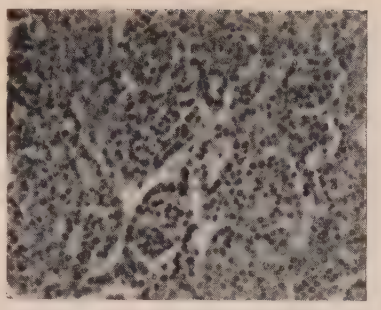


FIG. 1.
An area of the spontaneous tumor which occurs in a strain of Swiss mice. Note the general cellular anaplasia with few areas of adenomatous tendency. ×225.

*Methods and materials.* In a strain of Swiss mice a type of spontaneous tumors, presumably mammary in character, occurs in females.[6] The tumors may appear almost

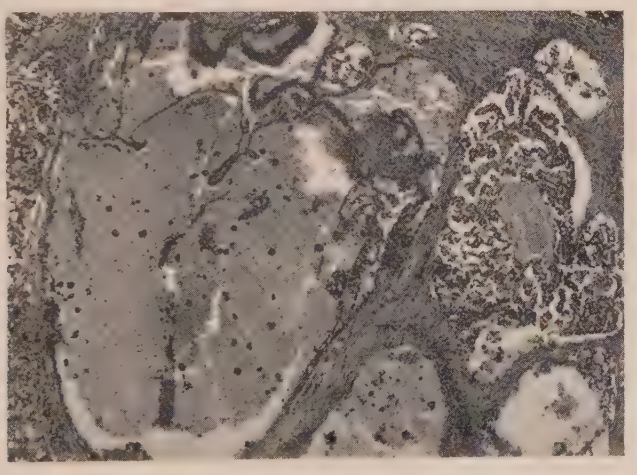


FIG. 2.
In such tumor one frequently encounters large vascular sinusoids with marked congestion. These spaces, however, are discrete and appear unruptured. ×100.

2 Smith, O. W., and Smith, G. V., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 116.

3 Tanturi, C. A., Canefa, J. F., and Banfi, R. F., *Revista "Medicina,"* 1945, **6**, 143.

4 Ludford, R. J., *Proc. Roy. Soc.*, ser. B., 1929, **104**, 493.

5 Duran-Reynals, F., *Am. J. Cancer*, 1939, **35**, 98.

6 Dunn, Thelma B., Mammary Tumors in Mice by Members of the Staff of the National Cancer Institute, American Association for the Advancement of Science, Smithsonian Institution Building, Washington 25, D.C., 1945, pp. 13-38.

TABLE I.
Effect of Necrosin on Spontaneous Tumor in Swiss Mice (3 mice were $C_3H$; 3 mice belonged to dba strain).

| No. of mice | No. of mice with frank hemorrhagic necrosis in tumor or in lung metastasis, or necrotic material in tumor | % of mice with frank necrosis in tumor, in metastases, or in both |
|---|---|---|
| 73 mice inj. with necrosin (1 to 8 inj. subcut.; 0.2 μg to 1748 μg per inj.) | 67 (including the $C_3H$ and dba mice) | 91.8 |
| 31 non-inj. mice* | 7 | 22.6 |

Chemical analysis of one sample of necrosin utilized yielded the following data:

| | % |
|---|---|
| Carbon | 41.24 |
| Hydrogen | 6.32 |
| Nitrogen | 11.58 |
| Sulfur | 1.56 |

* At times very old non-injected mice with very vascular spontaneous tumors display at necropsy variable amounts of necrosis in the tumor, with occasional necrosis likewise occurring in some of the metastases in the lungs. This suggests that canine necrosin administration hastens the time of necrosis to occur in the neoplasms studied.

anywhere in the subcutaneous tissue, but they are more prevalent in the ventral surface of the animal. The tumors are occasionally found in the axilla, in the shoulder, and even in the dorsal surface of the neck. Sometimes these tumors appear in the lower portion of the pelvis. The tumor may show areas of anaplasia interspersed with other areas of adenomatous tendency (Fig. 1). The tumors are characterized by their great vascularity. The presence of large vascular sinusoids is frequently seen (Fig. 2). The tumor is essentially carcinomatous in appearance.

Swiss mice with such tumors, varying in their size, were injected subcutaneously, at a distance from the tumor, with a colloidal solution of necrosin. The amount of necrosin varied from the equivalent of 1748 μg by dry weight to 0.2 μg. The injections, however, were always done in the fluid state in amounts ranging from .05 cc to 0.2 cc. The injections were repeated at intervals of 24 hours or longer. A study of the effect on the tumors was made at 1 hour, 4 hours, 6 hours and 7 hours after the injection of necrosin, and also at the death of the animal. Death occurred usually, though not always, several days after the last injection of necrosin.

*Results.* The results on over 100 mice are assembled in Table I.† In 73 mice injected with necrosin 91.8% manifested various degrees of necrosis in the tumor substance. Frequently the necrosis was hemorrhagic in character (Fig. 3). The tumor substance became in large part replaced by the free oozing of dark bloody-like material. Only here and there tufts of the original neoplastic tissue remained. Sometimes, although perhaps not as frequently, a mucoid-like yellowish fluid was found instead of the usually relatively firm tumor substance. This was taken to be a type of necrosis. Rarely a caseous-like central necrosis was also apparent in the tumor substance.

In all this work the effect on the metastases was of real interest and perhaps even of greater significance than the effect on the tumor proper. The tumor most frequently metastasizes to lung where the metastases appear, in the gross, as translucent-like foci. Microscopically these foci appear as discrete carcinomatous areas (Fig. 4). The injection of necrosin is accompanied not only by necrosis in the tumor proper, but also by hemorrhagic necrosis in many of the metastatic foci in lung tissue (Fig. 5). The gross appearance of the tumor following necrosin administration is seen in Fig. 6. Microscopically the appearance of the tumor in a treated mouse is, as stated above, exemplified in Fig.

† Since the completion of this manuscript further studies on this subject have been continued. This now involves well over 150 mice in 20 independent series of experiments.

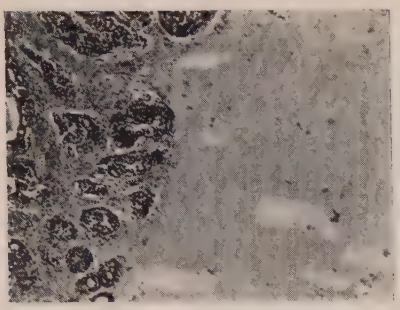


FIG. 3.
This animal received 3 subcutaneous injections of 0.5 cc of necrosin diluted with saline 1:5. Over 2 weeks later the animal died. A large amount of hemorrhagic fluid replaced in large part the tumor substance. ×75.

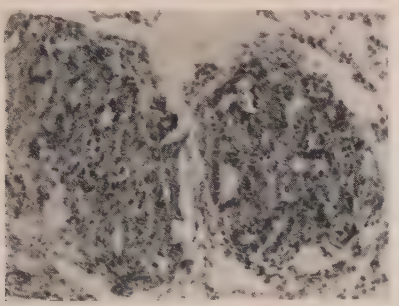


FIG. 4.
The spontaneous tumor of Swiss mice frequently metastasizes to lung. The illustration represents such metastasis. They appear as discrete carcinomatous areas. ×150.

3. The appearance, in the gross, of the tumor in a mouse treated subcutaneously with another globulin such as the leukocytosis-promoting factor of exudates of dogs[7] appears in Fig. 7. In brief, the leukocytosis-promoting factor of exudates seems to have no significant effect on the character of the tumor.

Necrosin is found to induce some necrotizing effect on the tumor as early as one hour or so following the subcutaneous injection of the material, in turn administered at a distance from the tumor. An effect can be elicited by the injection of as little as 0.2 $\mu$g of necrosin. The euglobulin fraction of normal canine blood serum usually fails to induce any effect on the tumor or on its metastases. Since necrosin contains a slight amount of proteolytic enzyme,[8] an attempt was made to determine the effect of papain. The injection of this enzyme seems to induce no significant visible effect. These studies, however, are being continued with other proteolytic enzymes.‡ Digestion of necrosin for several hours with either crystalline trypsin or papain does not seem to affect significantly the end result on the tumor. Necrosin administered to Swiss mice with spontaneous tumors does not appear to influence the longevity of the treated animal when compared with untreated mice. There may be several factors involved for this lack of effect on the life span. These are at present under investigation. One definite fact is a concomitant severe injury to the liver. This is illustrated in Fig. 8. The injury may be in the form of severe hepatic vacuolation. Sometimes varying degree of cellular infiltration is encountered. The kidneys at times reveal evident injury. A similar effect of necrosin was described in an earlier communication to occur in dogs.[1] Methods of obviating injury to the liver by necrosin are also being studied.

Some degree of necrosis is found in only 22.6% of the tumors of untreated mice (Table I). In such animals, however, the metastatic lesions of the lungs are seldom involved. It is for this reason that a study of the effect of necrosin on the metastatic lesions in the lung may perhaps yield more significant information than its effect on the tumor proper. Only several mice were available which belonged to different strains. These

[7] Menkin, Valy, *The Lancet*, May 17, 1947, pp. 660-662.

[8] Menkin, Valy, *Am. J. Physiol.*, 1946, **147**, 379.

‡ Since the completion of this manuscript, preliminary studies by the repeated subcutaneous injections of 0.5 mg of crystalline trypsin (Armour) in saline into Swiss mice with spontaneous tumors have yielded hemorrhagic necrosis in only some of the metastatic foci of the lungs. This would perhaps suggest a possible explanation of the action of necrosin, *i.e.*, through its slight degree of proteolytic property. This phase of the work is being studied further.

§ These studies are also being done on what has been found to be Strain A mice with apparently the same effect. This will, however, form the subject of a future communication.

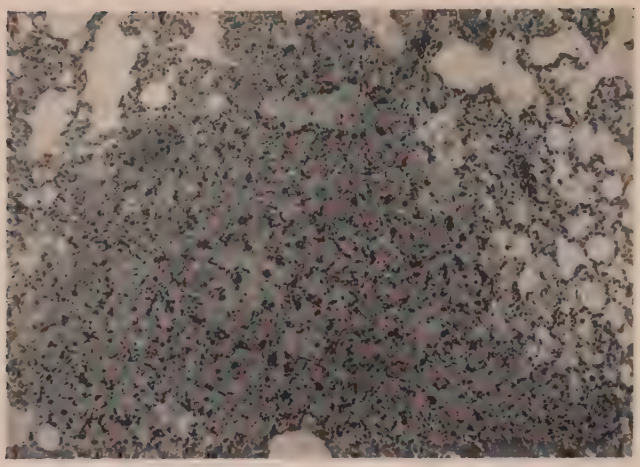


FIG. 5.
A metastatic focus in the lung of a Swiss mouse having received 3 subcutaneous injections of 0.5 cc of necrosin diluted with saline 1:5. Over 2 weeks later the animal succumbed. The metastatic area manifests considerable hemorrhagic necrosis. ×85.

FIG. 6.
Gross appearance of a spontaneous tumor in a Swiss mouse in turn injected subcutaneously twice with .05 cc of necrosin diluted with saline (1:5). The amount of necrosin was equivalent to 10 mg per injection. The animal was sacrificed one day following the second injection. Note the marked degree of hemorrhagic necrosis.

FIG. 7.
Gross appearance of a spontaneous tumor in a Swiss mouse following the subcutaneous injection of 0.1 cc of canine leukocytosis-promoting factor ($\alpha$ 1 and $\alpha$ 2 globulins of exudates). The mouse died 5 days after administration of the globulin material. There is no evidence of any necrosis in the tumor.

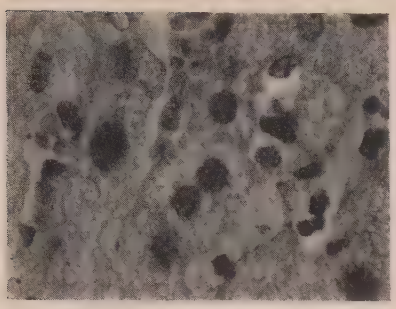


FIG. 8.
Appearance of the liver in a mouse injected subcutaneously twice with .05 cc of necrosin. The mouse succumbed 4 days following the second injection. Note the severe degree of injury to the liver in the form of marked vacuolation within the hepatic cells. ×560.

were C H and dba mice.§ Repeated subcutaneous injections of necrosin were[3] likewise followed by some degree of tumor necrosis with concomitant necrotizing effect on some of the lung metastasis. In these animals, however, many more injections of this toxic material were necessary, and the effect was generally not as pronounced as on the Swiss strain of mice. The extensive initial vascularity of the tumor has probably something to do with the ultimate effect of necrosin administration. Finally, in view of Shear's[9] recent work on bacterial polysaccharides necrosin was tested for the Molisch reaction, and it was found on a sample utilized to be negative. Culturing necrosin for bacteria in broth yielded negative results within eleven hours, and negative growth on agar slants within 24 hours. The necrosin samples employed ranged from fresh material (only several hours old) to material prepared over 2 years previously and preserved in a refrigerator. The effects obtained were identical. Finally, the subcutaneous injections of 0.2 cc of a slightly acid exudate (pH 6.9 to 7.0), in which presumably necrosin was present, induced hemorrhagic necrosis in the tumor and in some of the metastatic foci of Swiss mice.‖

*Conclusions.* Necrosin is a substance associated with the euglobulin fraction of usually acid inflammatory exudates. Its liberation by injured cells offers a reasonable explanation for the basic pattern of injury in inflammation.

The subcutaneous injections of canine necrosin, at a distance from the site of spontaneous tumors, in a strain of Swiss mice produce, after varying intervals, hemorrhagic or other types of necrosis in the tumor substance.

A necrotizing effect is also seen to occur in some of the metastatic lesions encountered in the lung.

Necrosin injections in these mice induce severe injury to the liver.

None of the effects on the tumor or on its metastasis are usually encountered with the use of the euglobulin fraction of canine blood serum, the leukocytosis-promoting fraction of canine exudates, or with one of the proteolytic enzymes adequately studied, namely papain.

9 Shear, M. J., *J. National Cancer Inst.*, 1944, **4**, 461.

‖ My thanks are due to Mrs. Ruth Lewin for technical aid in the course of this study.

16757

## Effect of Adrenocorticotrophic Hormone on Anaphylaxis in the Guinea Pig.

JACQUES LEGER,* W. LEITH AND BRAM ROSE. (Introduced by J. S. L. Browne.)
*From the McGill University Clinic, Royal Victoria Hospital, Montreal, Quebec.*

Adrenal cortical hormones are known to cause dissolution of lymphoid tissue,[1,2] and it has recently been shown that in sensitized animals, the rate of lymphocyte disintegration is paralleled by an increase of circulating antibodies.[3-6] This phenomenon may be elici-

* Fellow National Research Council, Canada.

1 Dougherty, T. F., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 132.

2 Selye, H., *J. Clin. Endocrinol.*, 1946, **6**, 117.

TABLE I.
Influence of A.C.T.H. on Anaphylactic Shock.

| Animals | Sensitizing dose, cc | Incubation period, days | Shocking dose, cc | A.C.T.H. mg | Symptoms | % shock |
|---|---|---|---|---|---|---|
| | | | Control group. | | | |
| 1 | .25 | 12 | 0.5 | | Severe | |
| 2 | .25 | 12 | 0.5 | | Fatal | |
| 3 | .25 | 12 | 1.0 | | ” | |
| 4 | .25 | 12 | 1.0 | | ” | |
| 5 | .25 | 12 | 1.0 | | ” | 12 died |
| 6 | .25 | 12 | 1.0 | | ” | |
| 7 | .25 | 12 | 1.0 | | ” | |
| 8 | .25 | 17 | 0.5 | | ” | |
| 9 | .25 | 17 | 0.5 | | Mild | 100% shock |
| 10 | .25 | 17 | 0.5 | | Fatal | |
| 11 | .25 | 17 | 0.5 | | Severe | |
| 12 | .25 | 17 | 0.5 | | Fatal | |
| 13 | .25 | 17 | 0.5 | | Severe | |
| 14 | .25 | 17 | 0.5 | | Fatal | |
| 15 | .25 | 17 | 0.5 | | Mild | |
| 16 | .25 | 17 | 0.5 | | Fatal | |
| 17 | .25 | 17 | 0.5 | | ” | |
| | | | Experimental group. | | | |
| 1 | .25 | 13 | 1.0 | 4 | Fatal | |
| 2 | .25 | 13 | 0.5 | 4 | ” | |
| 3 | .25 | 13 | 1.0 | 4 | Severe | |
| 4 | .25 | 13 | 1.0 | 4 | Fatal | |
| 5 | .25 | 13 | 1.0 | 4 | ” | 12 died |
| 6 | .25 | 13 | 1.0 | 4 | Mild | |
| 7 | .25 | 13 | 1.0 | 4 | Fatal | |
| 8 | .25 | 13 | 0.5 | 3.5 | ” | |
| 9 | .25 | 17 | 0.5 | 3.5 | Severe | 100% shock |
| 10 | .25 | 17 | 0.5 | 3.5 | Fatal | |
| 11 | .25 | 17 | 0.5 | 3.5 | Mild | |
| 12 | .25 | 17 | 0.5 | 3.5 | Fatal | |
| 13 | .25 | 17 | 0.5 | 3.5 | Severe | |
| 14 | .25 | 17 | 0.5 | 3.5 | Mild | |
| 15 | .25 | 17 | 0.5 | 3.5 | Fatal | |
| 16 | .25 | 17 | 0.5 | 3.5 | ” | |
| 17 | .25 | 17 | 0.5 | 3.5 | ” | |
| 18 | .25 | 17 | 0.5 | 3.5 | ” | |

Mild shock denotes symptoms of dyspnea, sneezing, diarrhea and restlessness.
Severe shock denotes symptoms of dyspnea going on to convulsions and unconsciousness.
Fatal refers to death.

ted by administration of adrenal cortical hormones, or by stimulation of the adrenal cortex by means of A.C.T.H. (adrenocorticotrophic hormone). The following investigation was carried out in order to study the effect of A.C.T.H. on anaphylactic shock in the guinea pig.

*Methods.* Thirty-five male and female guinea pigs weighing between 200 - 400 g were injected intraperitoneally with 0.25 cc of undiluted horse serum. These animals were submitted to a re-injection of ½ to 1 cc of the same antigen after an incubation period varying between 12 to 17 days. The readministration was performed via the jugular vein, which had been exposed by dissection in the unanaesthetized animal. The animals were divided into two groups, Group I comprising 17 control guinea pigs, and Group II comprising the remaining 18 animals. The animals of Group II were given 3½ to 4 mg of A.C.T.H. (s.c.) 6 to 8 hours prior to the shocking dose of the antigen. Since Dougherty and his colleagues used 1

[3] Dougherty, T. F., Chase, J. H., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 295.

[4] Dougherty, F. F., White, A., and Chase, J. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 28.

[5] Dougherty, T. F., Chase, J. H., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 135.

[6] Dougherty, T. F., and White, A., *J. Lab. and Clin. Med.*, 1947, **32**, 584.

mg A.C.T.H. for mice, 60 to 80 days of age and 10 mg of A.C.T.H. for 4-month-old rabbits, it was considered that weight for weight 3½ to 4 mg of A.C.T.H. would be a suitable dose for the guinea pigs used. The animals were carefully observed for the appearance of anaphylactic shock which was graded as follows: mild shock, severe shock, and death.

*Results.* The results of this experiment are summarized in Table I. The animals in both groups showed a similar response to the shocking dose of the antigen. Group I, the control group, all showed varying degrees of shock, which was fatal in 12 of 17 animals, with 3 showing severe shock and 2 showing symptoms of mild shock. Group II, the experimental group, also showed 100% reactivity, there being 12 instances of fatal shock, 3 cases of severe shock, and mild shock in 3 animals. The time of death occurred at approximately the same time interval following the shocking dose in both groups of animals. Therefore, one may conclude that A.C.T.H. in the dose administered, and under these experimental conditions, does not seem to have any effect on anaphylactic shock as elicited in guinea pigs by the procedure outlined above.

*Summary.* The administration of adrenocorticotrophic extract to sensitized guinea pigs prior to injection of a shock dose of antigen failed to influence the course of anaphylactic shock in a group of 18 animals.

We wish to thank Dr. J. F. Mote of Armour Laboratories for the preparation of A.C.T.H., and Mr. Feith for technical assistance. The horse serum was kindly supplied by Prof. L. Forte of the Universite de Montreal.

16758

## *In vitro* Studies of Aureomycin, a New Antibiotic Agent.*

ELEANOR A. BLISS AND CAROLINE A. CHANDLER. (Introduced by P. H. Long.)
*From the Departments of Preventive Medicine and Pediatrics, The Johns Hopkins University School of Medicine, Baltimore, Md.*

Samples of a new antibiotic, designated as A377 and later named aureomycin, were received from the Lederle Laboratories Division of the American Cyanamid Company, in November 1947. Since preliminary trials showed that the product had considerable activity *in vitro* against both a gram negative bacillus and a gram positive coccus, it was decided to test it further.

*Description.* According to information received from the Lederle Laboratories, aureomycin is derived from a strain of *Streptomyces aureofaciens.* The partially purified hydrochloride which was sent us is a bright yellow, fluffy, crystalline material. A 10% solution can be prepared in distilled water but the solubility is lowered by the presence of sodium chloride. The pH of a 0.1% aqueous solution is approximately 4. Dilute broth solutions pass through Berkefeld N and Seitz filters with but little loss of activity.

*Range of Action.* The least amount of aureomycin which prevents visible growth for 24 hours was determined for a variety of gram positive and gram negative bacteria. Polymyxin[1] and penicillin were used as standards of comparison in parallel tests. Serial 2-fold dilutions of the drugs in Difco Heart Infusion Broth, with 0.075% dextrose added, were inoculated with equal volumes of 18 hour old cultures so diluted as to result in approximately 200,000 organisms per cc. The diluent for the gram negative bacilli was

* These investigations were supported by grants from Abbott Laboratories, Eli Lilly and Company, Lederle Laboratories Division of the American Cyanamid Company, Parke, Davis and Company, and The Upjohn Company.

[1] Stansly, P. G., Shepherd, R. G., and White, H. J., *Bull. Johns Hopkins Hosp.*, 1947, **81**, 43.

TABLE I.
Comparative Activity of Aureomycin (A377), Penicillin, and Polymyxin (B71) for Various Bacteria.

| Bacterium | Group or type | Medium | Inoculum, cc | Minimal inhibitory conc., γ/cc (24 hr) A377 | Peni. | B71 | A/P ratio |
|---|---|---|---|---|---|---|---|
| *Str. hemolyticus* | A-C203 | Blood HIB | .5 × 10-3 | .3125 | .006 | | 50 |
| ,, ,, | D-Zymog* | ,, | ,, | 1.25 | 2.5 | >(20) | .5 |
| ,, ,, | F-For | ,, | ,, | .625 | .05 | | 12.5 |
| ,, ,, | G-Dog | ,, | ,, | 1.25 | .0125 | | 100 |
| *D. pneumoniae* | I SV1 | ,, | ,, | .3125 | .025 | | 12.5 |
| ,, ,, | I Bailey | ,, | ,, | .3125 | .0125 | | 25 |
| ,, ,, | II | ,, | × 10-4 | .3125 | | | |
| ,, ,, | III | ,, | ,, | .156 | | | |
| *Str. faecalis* | D-Tarr* | ,, | × 10-3 | 1.25 | >1 | | < 1 |
| *Staphylococcus* | Barlow | ,, | ,, | .625 | .025 | | 25 |
| (all are *aureus*) | Zeut | ,, | ,, | .62 | .05 | | 12.5 |
| | Zorn | ,, | ,, | .62 | .05 | | 12.5 |
| *B. subtilis* | Lederle | ,, | ,, | .08 | | | |
| ,, ,, | FDA | ,, | ,, | 2.5 | | | |
| ,, ,, | ,, | Plain HIB | ,, | 2.5 | | | |
| *E. coli* | 4 S1 | ,, | × 10-4 | 5.0 | | .156 | 32 |
| ,, ,, | 9 | ,, | ,, | 5.0 | | .156 | 32 |
| *A. aerogenes* | 7 | ,, | ,, | 5.0 | | .3125 | 16 |
| ,, ,, | 8 | ,, | ,, | 5.0 | | .625 | 8 |
| *K. pneumoniae* | Lederle | ,, | ,, | 1.25 | | .156 | 8 |
| ,, ,, | Cephus | ,, | ,, | 5.0 | | .625 | 8 |
| *Ps. aeruginosa* | 16 | ,, | ,, | >20 | | 2.5 | > 8 |
| ,, ,, | Calloway | ,, | ,, | >20 | | 2.5 | > 8 |
| *Proteus* | 17 | ,, | ,, | >20 | | >20 | |
| ,, | 18 | ,, | ,, | >20 | | >20 | |
| *H. influenzae* | b-Pittman | Fildes-HIB | — | 2.0 | | .35 | 6 |

* Four additional strains of Group D streptococci ($\alpha$ and $\beta$) were tested. All were inhibited by 1.25 γ/cc A377 and were less sensitive to penicillin.

heart infusion broth, that for the cocci contained 4% of washed rabbit red cells. The tests were read after 18 hours incubation at 37°C.

The results are shown in Table I. It is apparent that, in the test tube and with the inocula used here, aureomycin is less effective than polymyxin and penicillin against the gram negative bacilli and the gram positive cocci, respectively. The only exception noted was in the case of the Group D streptococci. Both $\alpha$ and $\beta$ strains of these organisms were more susceptible to aureomycin than to penicillin. In the concentrations tested here, neither aureomycin nor polymyxin checked the growth of *Proteus*.

Comparison was also made with streptomycin in a few instances, with the results shown in Table II. Aureomycin was less effective than streptomycin in suppressing, for 18 hours, the growth of *E. coli* and *K. pneumoniae*. The strain of staphylococcus which was used in this test was more susceptible to the new agent than to streptomycin, while the two agents were equally effective against *H. influenzae b*.

*Bacteriostatic vs. Bactericidal Activity.* In the course of the above tests it was noted that on further incubation the end-points for aureomycin moved up day by day. Moreover, subcultures on blood agar plates made after the first day of incubation showed the presence of

TABLE II.
Comparative Activity of Aureomycin (A377) and Streptomycin for Various Bacteria, at the End of 24 and 48 Hours Incubation.

| Organism | Drug | End-point—γ/cc: Turbidity 24 hr | Turbidity 48 hr | Subculture 24 hr | A/S ratio 24 hr |
|---|---|---|---|---|---|
| *E. coli* | A377 | 10.0 | 20.0 | >30.0 | 2.0 |
| | Streptomycin | 5.0 | 5.0 | 5.0 | |
| *Staph. aureus* | A377 | 1.0 | 10.0 | >20.0 | 0.1 |
| | Streptomycin | 10.0 | 10.0 | 10.0 | |
| *K. pneumoniae* | A377 | 2.5 | 10.0 | 15.0 | 2.0 |
| | Streptomycin | 1.25 | 1.25 | 1.25 | |
| *H. influenzae* | A377 | 2.0 | 2.0 | 2.0 | 0.67 |
| | Streptomycin | 3.0 | 3.0 | 3.0 | |

Note: Medium—Heart infusion broth, except in case of *H. influenzae* where Fildes broth was used.

TABLE III.
Growth Rate of *E. coli* and Beta Streptococcus in the Presence of Aureomycin (A377), Polymyxin (B71), and Penicillin.
(a) *E. coli*—Heart Infusion Broth.

| Time of plating Hr after inoculation | Broth control | A377 20 γ/cc* | B71 0.2 γ/cc‡ |
|---|---|---|---|
| | Organisms per cc | | |
| | × 1000 | × 1000 | × 1 |
| 0 | 220 | 230 | 310,000 |
| 1 | 430 | 90 | 20 |
| 3 | 16,000 | 35 | <10 |
| 6 | 300,000 | 12 | < 2 |
| 24 | 5,000,000 | 13 | < 2 |
| 72 | — | ++++† | — |

(b) Beta Streptococcus C203—Heart Infusion Broth + Blood.

| | Broth control | | A377 1.25 γ/cc* | | Penicillin 0.025 γ/cc* | |
|---|---|---|---|---|---|---|
| | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 |
| | Organisms per cc × 1000 | | | | | |
| 0 | 140 | 275 | 40 | 305 | 120 | 290 |
| 1 | 200 | 545 | 90 | 95 | 40 | 30 |
| 3 | 4000 | — | 42 | — | 5 | — |
| 4 | — | 19,000 | — | 12 | — | .3 |
| 5 | 115,000 | — | 23 | — | 1.8 | — |
| 24 | 550,000 | — | 6 | — | .03 | — |
| 48 | — | ++++ | — | ++++ | — | 0 |
| 72 | — | — | ++++ | — | 0 | — |

* 4 times minimal inhibitory concentration for test organism.
† Maximum turbidity.
‡ This test was run on another occasion; 0.2 γ/cc polymyxin is only slightly more than the minimal inhibitory concentration for *E. coli*.

viable organisms in tubes containing 20 times the amount of antibiotic which had prevented visible growth.

These observations, illustrated in Table II, indicated that aureomycin is bacteriostatic rather than bactericidal in its action. This point has been confirmed by studies of the rate of growth of *E. coli* and the C203 strain of hemolytic streptococcus in the presence of 4 times the inhibitory concentration of the agent. In the case of C203 comparison was made with penicillin. Ten cc volumes of the

TABLE IV.
Deterioration of Aureomycin (A377) after Incubation Alone and in Presence of Test Organism.

| Duration of preliminary incubation, hr | Visible end-point γ/cc | | |
|---|---|---|---|
| | 24 hr reading | 48 hr reading | 72 hr reading |
| * | | | |
| 0 | 1.25 | 5.0 | 10 |
| 24 | 10 | 20 | 20 |
| 48 | 20 | >20 | >20 |
| † | | | |
| 0 | 1.25 | 2.5 | 10 |
| 24 | 2.5 | 10 | 20 |
| 48 | 5 | 20 | >20 |

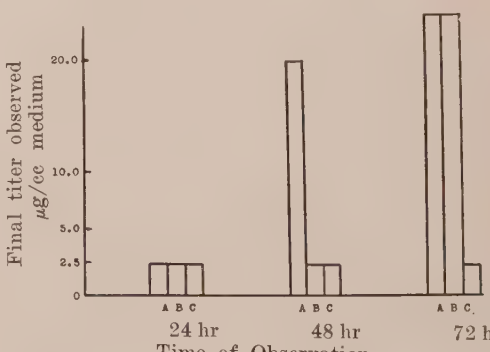
* 40 γ/cc A377 in heart infusion broth after 0 hr, 24 hr, 48 hr incubation—titrated against test organism (*Staph. aureus*).

† Supernatant of 40 γ/cc A377 after incubation with live *Staph. aureus* at 0 hr, 24 hr, 48 hr—titrated against test organism (*Staph. aureus*).

solutions were inoculated with 0.1 cc of 1:100 dilutions of the cultures. Plates were poured at intervals. The results, shown in Table III a and b, indicate that although aureomycin brings about a progressive decline in the bacterial population for the first 24 hours of exposure, the organisms eventually recover and reach maximum growth. In contrast, penicillin caused an immediate decline in the bacterial count which terminated in the sterilization of the cultures. Polymyxin, in a concentration only slightly above the minimal inhibitory concentration, has been observed, in other studies,[2] to exert an extremely rapid bactericidal effect upon *E. coli*. Aureomycin is clearly less decisive in its action than are penicillin and polymyxin.

It is not known at present whether the eventual outgrowth, in the case of aureomycin, is the result of the survival of resistant organisms or is due to the deterioration of the drug. As shown in Table IV, when aureomycin was titrated in the usual manner after incubation at 37°C in broth alone or in the presence of the test organism for 24 and 48 hours, there was progressive and rapid inactivation.

Confirmatory evidence of this phenomenon was obtained in another set of experiments in which *K. pneumoniae* was the test organism. Three sets of tubes, each containing 2-fold dilutions of aureomycin from 0.625 γ/cc through 20 γ/cc were inoculated in the usual manner. After 24 hours incubation, growth was found to have been inhibited by 2.5 γ/cc per tube of drug in all 3 sets of tubes. The first set (a) was allowed to incubate without further addition of drug through 72 hours. Fresh drug, to give an additional 2.5 γ/cc per tube, was added to the second set (b) at 24 hours, and to the third set (c) at 24 and 48 hours. As shown in Fig. 1, the inhibitory concentration in (a) was 20 γ/cc at 48 hours and more than 20 γ/cc at 72 hours. Growth in (b) was inhibited by 2.5 γ/cc for 48 hours and then titrated to more than 20 γ/cc at 72 hours. In (c), however, with drug replenished at 24 and 48 hours, the original inhibitory titre of 2.5 γ/cc was maintained throughout the 72 hour observation period. With no further addition of drug to set (c) at 72 hours, growth occurred through 20 γ/cc at 96 hours.




A: No addition of aureomycin.
B: Addition of 2.5 μg/cc at 24 hr.
C: Addition of 2.5 μg/cc at 24 and 48 hr.

FIG. 1.

Effect of adding increments of fresh aureomycin.

2 To be published.

TABLE V.
Effect of Normal Human Serum on the End-points of Titrations of Aureomycin.

| Bacterium | Medium | Serum concentration, % 50 | 25 | 12½ | 6¼ | 3⅛ | 1½ | 0 |
|---|---|---|---|---|---|---|---|---|
| | | Minimal inhibitory conc. of A377, γ/cc | | | | | | |
| *E. coli* | S1G1 | 25 | 12.5 | 6.25 | 6.25 | 5 | 1.56 | 1.25 |
| | HIB | 80 | 15 | 10 | | | | 4 |
| Strept. C203 | HIB + Blood | 6.25 | 1.25 | 0.625 | 0.312 | 0.312 | 0.125 | 0.125 |

TABLE VI.
Instability of Aureomycin to Heat. Minimal Inhibitory Concentrations for *E. coli* after Various Exposures.

| Duration of exposure | Temperature, centigrade 10 | 24 | 37 | 56 |
|---|---|---|---|---|
| | Minimal inhibitory conc., γ/cc | | | |
| 0 min. | | | 6.25 | 6.25 |
| 25 " | | 6.25 | | |
| 40 " | | | | 6.25 |
| 1 hr | | | | 12.5 |
| 2½ " | | 6.25 | | |
| 4 " | | | | 25.0 |
| 5 " | | 6.25 | | |
| 18 " | 6.25 | | 20 | |
| 24 " | 6.25 | 12.5 | | |

*Effect of Environmental Factors on Titration. Medium.* In contrast to that of the sulfonamides, the activity of aureomycin for *E. coli* is but little better in the synthetic medium, S1G1,[3] than it is in heart infusion broth. The addition of serum, however, to either medium has a marked effect upon the titration end point. As shown in Table V, the end points vary directly with the concentration of serum. In this respect aureomycin resembles penicillin. The effect, however, is very much greater on the new antibiotic, 50% serum causing a 20- to 50-fold raising of the end-point of aureomycin and only a 4-fold increase in the case of penicillin.

*Temperature.* Aureomycin showed progressive loss of activity when held, in broth solutions, at higher than refrigerator temperatures. As shown in Table VI, at room temperature there was no change for 5 hours, but by 24 hours half the activity had been lost. Storage at 37°C for 18 hours before use reduced activity by 60%. At 56°C the rate of deterioration increased; 50% of the activity was lost in 1 hour, 75% in 4 hours.

*Relation of Size of Inoculum to Minimal Inhibitory Concentration.* Seven sets of serial 2-fold dilutions of aureomycin, containing 0 to 50 γ/cc, were set up in Wassermann tubes. Each set was inoculated with one of a series of 10-fold dilutions, in blood broth, of an 18 hour culture of C203. The tests were incubated at 37°C for 18 hours. It was found that the end-point depends, to a certain extent, upon the number of organisms inoculated. Inocula of $10^{-5}$ to $10^{-7}$ cc of culture were inhibited by 0.156 γ/cc aureomycin, while 0.312 γ/cc was required to delay the development of $10^{-2}$ to $10^{-4}$ cc. The end-point obtained with 1/10 cc of culture could not be determined because there was sufficient streptolysin present in the inoculum to hemolyze the red blood cells.

*Summary.* A new antibiotic, aureomycin, is effective *in vitro* against both gram positive and gram negative bacteria. Although its activity is of a lower order than that of penicillin for gram positive cocci or polymyxin for gram negative bacilli, it is effective against both classes of bacteria in concentrations close

[3] Bliss, E. A., and Long, P. H., *Bull. Johns Hopkins Hosp.*, 1941, **69**, 14.

to those required of streptomycin. Inhibition at such concentrations, however, is fleeting in the case of aureomycin, much larger amounts being required for permanent suppression of growth. The new agent is bacteriostatic rather than bactericidal in its effect. Its antibacterial action is greatly diminished in the presence of serum, *in vitro*, and deterioration at room temperature is marked. Differences in the size of the inoculum have a moderate effect upon the minimal inhibitory concentration.

The authors are grateful to Miss Elizabeth Burr and Miss Jean Fisher of this department for technical assistance, and to Miss Minnie Schreiber and Miss Rosemary Stokes, of the Biological Division, The Johns Hopkins Hospital, for providing many of the test strains.

16759

## Further Studies in Experimental Allergic Encephalitis in the Guinea Pig.

HILARY KOPROWSKI AND GEORGE A. JERVIS. (Introduced by Herald R. Cox.)
*From the Section for Viral and Rickettsial Research, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y., and the Research Department, New York State Department of Mental Hygiene, Letchworth Village, Thiells, N. Y.*

It has been indicated[1,2] that an encephalitic process, presumably allergic in nature, results in guinea pigs inoculated with rabbit-brain tissue and "adjuvants". A brief report is herein given of additional experiments with brain-tissue inocula derived from various animal species, as well as on the effect of progressive dilutions of brain-tissue inocula, attempts at purifying the antigen, effect of intracerebral re-injection of the antigen, and on the development of antibrain antibodies in animals inoculated either subcutaneously alone, or subcutaneously and then followed by an intracerebral inoculation of the brain suspension.

1. *Inoculation of brain tissue from various animal species.* The technic followed was the same as reported previously[2] except that brain material from 5 different species was used for the subcutaneous inoculation. Paralysis of inoculated guinea pigs was observed usually 21 or more days after the injection. Death followed from 1 to 15 days after onset of paralysis and occasionally occurred in animals which had shown no neurological manifestations. The results obtained (Table I) do not lend themselves to a satisfactory comparison because of the low and unequal number of animals used in each group. In general, the percentage of animals which succumbed was proportionately similar in each group regardless of the source of the brain tissue used in the inoculum.

Lesions in the central nervous system (CNS), of the type already described,[2] were observed in all animals. In general no conspicuous differences were noted in the lesions which could be ascribed to the use of heterologous antigens, but in some guinea pigs more extensive lesions were obtained when guinea pig brain suspensions were used as inoculum.

2. *Effect of progressive dilutions of brain tissue inoculum.* Phenolized rabies vaccines prepared from calf and rabbit brain tissue, respectively, were used as source material for the inoculum. Starting with the 1:20 dilution of brain emulsion, serial twofold dilutions were made in saline, incorporated into adjuvants and injected into guinea pigs. For the sake of brevity, the results of the experiment (Table II) were grouped irrespective of the brain tissue used as inoculum. For comparison are included data summarizing the results of various experiments in which guinea pigs were inoculated with a suspension of normal rabbit brain tissue in 1:10 dilution. Up to and including the 1:80 dilution, there appeared to be no material difference in the

[1] Freund, J., Stern, E. R., and Pisani, T. M., *J. Immunol.*, 1947, **57**, 179.

[2] Jervis, G. A., and Koprowski, H., *J. Neuropath. and Exp. Neurol.*, 1948, **7**, 309.

TABLE I.
Results Obtained in Guinea Pigs Injected with Brain-tissue Inocula Derived from Various Animal Species.

| Brain tissue used | Guinea pigs inoculated, No. | Guinea pigs | | | |
|---|---|---|---|---|---|
| | | Paralyzed | | Died | |
| | | No. | % | No. | % |
| Rabbit | 94 | 45 | 48 | 39 | 41 |
| Guinea pig | 20 | 11 | 55 | 10 | 50 |
| Human | 20 | 7 | 35 | 11 | 55 |
| Calf | 19 | 9 | 47 | 7 | 37 |
| Swine | 9 | 6 | 66 | 6 | 66 |
| Total | 162 | 78 | 48 | 73 | 45 |

TABLE II.
Results Obtained in Guinea Pigs Inoculated with Various Dilutions of Rabies Vaccines.

| Dilution of brain tissue | Guinea pigs inoculated, No. | Guinea pigs | | | |
|---|---|---|---|---|---|
| | | Paralyzed | | Died | |
| | | No. | % | No. | % |
| 1:20 | 19 | 9 | 47 | 6 | 32 |
| 1:40 | 15 | 10 | 66 | 10 | 66 |
| 1:80 | 19 | 10 | 52 | 13 | 68 |
| 1:160 | 20 | 4 | 20 | 5 | 25 |
| 1:320 | 19 | 1 | 5 | 2 | 10 |
| 1:10* | 162 | 78 | 48 | 73 | 45 |

* Normal rabbit-brain tissue used as inoculum.

number of paralyzed or dead animals, and the dilution used. However, a definite decline in the incidence of paralysis or death was observed among the animals inoculated with the 1:160 and 1:320 dilutions.

3. *Attempt at purification of brain antigen.* Precipitation with methanol[3] failed either to enhance or to inhibit the antigenic property of the human brain substance and further fractionation was done by Klenk's technic.[4] The methanol-chloroform extracted fraction, which contains cerebrosides and sphyngomyelin, caused paralysis in 2 animals of 10, while 3 of 10 animals showed paralysis following the injection of the protein fraction remaining after extraction of all lipoids. The pathological picture differed in no way from that obtained with the whole brain tissue.

The results obtained with the lipoid fraction conform to expectation since it has been shown that the substance responsible for the allergic reaction is present in the myelin sheaths[5,6] which have a high cerebroside content. Alvord[6] has recently reported that the phosphatide fraction obtained with Bloor's technic is also effective in producing allergic encephalitis. Apparently the antigenic property is shared by more than one substance present in the white matter of the CNS. It should be noted, however, that the fractions used are not chemically pure substances but only mixtures of cerebrosides and sphyngomyelins, or of lecithins and cephalins, containing many impurities. The purification and identification of the antigen awaits further study and elucidation.

4. *Intracerebral re-inoculation of the antigen.* Five to 7 weeks after the primary subcutaneous injection of brain antigen combined with adjuvants, guinea pigs which had survived were given intracerebrally 0.1 ml of

[3] Koprowski, H., Black, J., and Cox, H. R., *Proc. IVth Internat. Congr. Microbiol.*, Copenhagen, 1947, in press.

[4] Klenk, E., *Hoppe-Seyler Z., f. physiol. Chem.*, 1939, **262**, 128.

[5] Kabat, A. E., Wolf, A., and Bezer, E. A., *J. Exp. Med.*, 1947, **85**, 117.

[6] Alvord, E. C., Jr., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 459.

TABLE III.
Results of Intracerebral Inoculation of Guinea Pigs Previously Sensitized by Subcutaneous Injection of Homologous or Heterologous Antigen.

| Brain tissue inoculated subcut. | Brain tissue used in subsequent intracerebral inoculation* | | | |
|---|---|---|---|---|
| | Rabbit | Guinea pig | Human | Calf |
| Rabbit | 17/19 | 1/2 | | |
| Guinea pig | 0/3 | 1/6 | | |
| Human | | | 9/9 | |
| Calf | 0/3 | 0/3 | | 3/3 |

* Denominator denotes the number of guinea pigs inoculated, numerator the number which died.

10% suspension of brain tissue in saline. With one exception, a high mortality ratio was obtained (Table III) in those groups which had received intracerebral inoculation of the same species-brain tissue suspension to which they had been previously sensitized. Death of the animals, occurring 3 to 15 days after the intracerebral inoculation, was usually preceded by signs of tremors, tonic spasms of the neck, paralysis, and occasionally by convulsions. The exception, mentioned above, occurred in the group of 6 animals which were injected both subcutaneously and intracerebrally with guinea pig brain. In this group only 1 of 6 animals died and its death may have been accidental since no marked pathologic changes were noted on examination of the brain tissues (see below). In the remaining groups of guinea pigs which had received intracerebrally brain tissue suspensions of animals other than from those to which they had been previously sensitized by subcutaneous inoculation, only 1 of 11 died.

Histopathological examinations[2] were made of the brain and spinal cord tissues of all guinea pigs that died, and of those which survived and had been sacrificed approximately 30 days after inoculation. Conspicuous changes were observed at the site of injection. In animals which had died within a few days after the inoculation, the local lesion consisted of a large necrotic hemorrhagic area. Only tissue-debris was seen at the center, while at the periphery large numbers of granular compound corpuscles and rich perivascular infiltrations of hematogenous elements were observed. In animals which died after the first week, hematogenous elements were scanty and infiltrations with compound granular elements predominated so that the local lesion was mainly one of demyelination with central necrosis. Furthermore, throughout the CNS, numerous disseminated areas of demyelination and perivascular infiltration of the type previously described[2] were observed. The fact that the animals had shown no conspicuous neurological signs before the intracerebral injection suggests that at least some of these lesions were of recent appearance and followed the intracerebral administration of antigen, and not older lesions occasioned by the subcutaneous injection. The pathological changes were most striking in those animals which received intracerebrally the same species-antigen to which they had been sensitized, and much less severe in those which had received antigen derived from two different species. It was, however, a difference in degree rather than in quality. The 6 animals (Table III) which were injected both subcutaneously and intracerebrally with guinea pig brain were an exception, since the local pathological lesion was mild.

Ten control animals which received intracerebral injections of brain tissue, without previous sensitization, showed no clinical signs and only minimal pathological lesions.

The focal necrotic lesions of the brain observed in these experiments bore a resemblance to those reported in experimental Arthus-phenomenon in the brain of rabbit,[7] guinea pig,[8] and monkey.[9] That the lesion

[7] Davidoff, L. M., Seegal, B. C., and Seegal, D., *J. Exp. Med.*, 1932, **55**, 163.

[8] Alexander, L., and Campbell, A. C. P., *Am. J. Path.*, 1937, **13**, 229.

was more marked when the intracerebral inoculum was from the same species as that injected subcutaneously, conforms to the hypothesis that the pathological changes are anaphylactic in nature. However, the process seems to be of uncommon intensity in these experiments as evidenced by the high mortality ratio. It is interesting to note that Freund[1] also observed high mortality after reinoculation of the brain substance intracutaneously into a sensitized animal. These findings may offer some indication as to the possible allergic nature of the encephalomyelitic process.

5. *Determination of antibrain antibodies in animals.* The presence of antibrain antibodies in the blood serum of guinea pigs was determined by complement-fixation tests against three antigens: human, normal guinea pig and rabbit brain which were prepared according to the technic of Casals and Palacios.[10] The technic of the test itself followed the one generally employed in this laboratory[11] except that 1.5 units of guinea-pig complement was used. Determinations were performed (a) in animals injected subcutaneously as described under items 1 and 3, and (b) in animals first injected subcutaneously and then intracerebrally as described under item 4.

(a) Complement-fixing antibodies against rabbit brain antigen were first noted on the 7th day after the subcutaneous injection of rabbit-brain suspension combined with adjuvants. On the 11th day, antibodies against brain antigens of all 3 species appeared, and the titer rose rapidly reaching a peak between the 16th and 21st day, followed by a gradual decline. In 2 of the 4 animals examined, antibodies were still present on the 193rd day after injection. With the rabbit-brain inoculum, the titer of antibodies in serum was always higher when tested against rabbit-brain antigen than against guinea pig or human antigen. In further experiments, it was noted than brain-specific antibodies appear in all injected animals irrespective of the species-source of the brain tissue inoculum. The only significant exception was the guinea pig brain antigen with which, while it induced marked incidence of paralysis in the inoculated animals (Table I), no antibrain antibodies were demonstrated. In general, no correlation was observed between the titer of antibrain antibodies and the clinical picture presented. Following the injection of rabbit-brain tissue, incorporated in Bayol F and lanolin but without the addition of acid fast bacilli, complement-fixing antibodies against rabbit brain were first observed on the 20th day after injection, a slight decrease in titer was seen on the 27th, and no antibodies were demonstrable on the 41st day. Against human brain, antibodies were observed at about the same time as antibodies against rabbit brain, but the titer of the former was much lower. No antibodies fixing guinea pig brain were detected at any time after inoculation. In animals injected with two human-brain fractions, as described under item 3, no antibodies against nonfractionated human brain and guinea-pig brain antigens were detected on the 13th day after injection, but 2 weeks later almost all of the guinea-pig sera showed titers ranging from 1:4 to 1:32 against human-brain antigen and slightly lower titers against guinea-pig brain antigen.

From the foregoing it seems reasonable to consider the appearance of complement-fixing antibodies as a constant phenomenon in guinea pigs injected with brain substance incorporated into adjuvants. This is in contrast to some previous studies in which inoculation of adjuvant-brain emulsions into guinea pigs failed to elicit antibodies,[12] or their presence was demonstrable only in 1 of 3 animals.[1] These antibodies are apparently not species-specific. The addition of acid fast bacilli to the emulsion of brain tissue definitely enhances formation of antibodies and broadens

9 Jervis, G. A., Ferraro, A., Kopeloff, L. M., and Kopeloff, N., *Arch. Neurol. and Psychiatr.*, 1941, **45**, 733.

10 Casals, J., and Palacios, R., *J. Exp. Med.*, 1941, **74**, 409.

11 Kolmer, J. A., and Boerner, F., Approved Laboratory Technic, Chap. 29, 3rd edition, Appleton Century Co., New York, 1941.

12 Kopeloff, L. M., and Kopeloff, N., *J. Immunol.*, 1947, **57**, 229.

their immunological pattern, but apparently it is not an indispensable factor. The formation of antibrain antibodies was described by several groups of investigators[13–16] who used as antigen brain tissue without adjuvants. However, inclusion of adjuvants in the inoculum seemed to facilitate the formation of antibodies.[17] The lack of any significant relation between the clinical status of the animal and the level of antibrain precipitins was noted also by Kopeloff and Kopeloff[12] in rabbits.

(b) Antibrain antibodies were determined in animals first injected subcutaneously and then intracerebrally, as described under item 4. In almost all, the titers of antibrain antibodies obtained after the subcutaneous inoculation showed an increase following the intracerebral inoculation. However, there appeared to be no correlation between the clinical status of the animals and the level of antibodies before and after the intracerebral inoculation. Guinea pigs which had shown no antibrain antibodies after the subcutaneous inoculation with guinea pig brain, did develop antibodies following the intracerebral inoculation of the same antigen. A rise in antibody level after intracerebral injection was noted also in those animals which had been injected subcutaneously with rabbit brain suspension combined with adjuvants but without *Mycobacterium tuberculosis*. In the control experiments, no complement-fixing antibodies were detected in 10 nonsensitized guinea pigs inoculated intracerebrally with 0.1 ml of 10% rabbit-brain suspension.

These serological findings, although not directly related to the clinical status of the animal, may be correlated with the pathological data reported above, which indicate that the difference in the histopathological lesions observed in sensitized guinea pigs that died or survived after the intracerebral inoculation was one of degree rather than quality. Possibly the increase in titer of antibrain antibodies is due to the release in the previously sensitized animal of small amounts of antigen from the brain tissue as a result of some microscopic injury suffered at the time of the intracerebral inoculation. However, this latter hypothesis requires further experimentation before any conclusions are warranted. The intracerebral "challenge" technic of previously sensitized guinea pigs, described above, may prove of value in the investigation of allergenic or paralitogenic properties of normal brain tissue or its fractions. This technic may be further broadened by injecting either non-fractionated homologous brain tissue, or purified fractions of the brain substance into animals previously sensitized by the subcutaneous route. Clinical observations of the animals, coupled with serological study, may throw additional light on the puzzling phenomenon of the allergenic properties of brain tissue.

In the discussions of the various aspects of the experiments reported above, reference to any possible application of the findings to human pathological conditions has been purposely avoided. Further experimental work is essential before one is justified to draw any conclusions applicable to conditions observed in human subjects, which could be attributed to the allergenic properties of brain tissue.

*Summary.* Allergic encephalitis in guinea pigs was produced by inoculation of brain tissue derived from 5 different mammalian species. Dilutions of phenolized rabies vaccines combined with adjuvants also caused paralysis of the inoculated animals. The majority of the guinea pigs previously sensitized by subcutaneous injection of brain tissue incorporated into adjuvants died after the intracerebral inoculation of homologous brain tissue (but not when brain tissue of cavian origin was used). Pathologic changes observed in the animals were described. Complement-fixing antibrain antibodies were found in sera of guinea pigs inoculated subcutaneously with brain tissue. Intracerebral reinoculation of these animals usually resulted in the rise of titer of the humoral antibrain antibodies.

[13] Witebsky, E., and Steinfeld, J., *Z. f. Immunitätsf. u. Exp. Therap.*, 1928, **58**, 271.

[14] Lewis, J. H., *J. Immunol.*, 1933, **24**, 193.

[15] Schwentker, F. F., and Rivers, T. M., *J. Exp. Med.*, 1934, **60**, 559.

[16] Bailey, G. H., and Gardner, R. E., *J. Exp. Med.*, 1940, **72**, 499.

[17] Kopeloff, L. M., and Kopeloff, N., *J. Immunol.*, 1944, **48**, 297.

16760

# Bactericidal Action of Streptomycin-Penicillin Mixtures *In vitro*.

Anna C. Nichols. (Introduced by I. S. Ravdin.)

*From the Harrison Department of Surgical Research, Schools of Medicine, University of Pennsylvania, Philadelphia.*

Studies were carried out in order to determine whether streptomycin and penicillin in combination were synergistic in their bactericidal action against 4 common organisms. Synergism is defined for the purpose of this paper as a cooperative action greater than would be predicted were the action of the agents mathematically additive.

*Methods.* On the basis of preliminary experiments, all media were adjusted to a sodium chloride concentration of 0.25% and to a pH of 7.6. All tests were performed in beef infusion broth (Difco) containing 1% neopeptone. The bacteria were grown at 37°C and a 6-hour culture was diluted so as to contain approximately 500,000 bacteria per/ml of media. One ml of this dilution was used as the inoculum.

Test organisms were *Staphylococcus aureus* Oxford H, *Staphylococcus aureus* No. 209, *Staphylococcus aureus* SM, and *Streptococcus hemolyticus* C-203 MV. All organisms were tested separately for susceptibility to penicillin and streptomycin. Titration of penicillin was made in 2 ml of medium and 1 ml of standard inoculum was added. The cultures were inoculated at 37°C. Readings were made at 24 hours compared with a bacterial control and subcultures were made of each tube to blood agar to determine the presence of growth. Streptomycin titrations were made in the same manner. This method determined the minimum lethal concentration of penicillin and of streptomycin for each organism. In each instance it was also determined that growth did occur in concentrations of 75% or more of this minimum lethal concentration.

Using the minimum lethal concentration as a guide, titrations of the combined antibiotics were set up. A penicillin titration was made to which varying amounts of streptomycin were added. Penicillin, streptomycin, and bacterial controls were set up with each titration. Subcultures were made to blood agar and compared with subcultures of the antibiotic control.

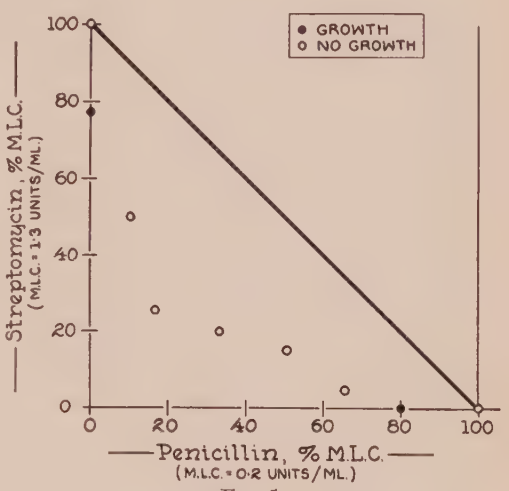



FIG. 1.
*Staphylococcus aureus* Oxford H.

*Experimental Results.* Fig. 1 is a typical graph for *Staphylococcus aureus* Oxford H. The minimum lethal concentration of penicillin was 0.2 unit per ml and for streptomycin was 1.3 units per ml. The diagonal line represents bactericidal combinations of penicillin and streptomycin which should be bactericidal if these two antibiotics were additive in their effect. For any given concentration of penicillin, the value representing the smallest amount of streptomycin which rendered the mixture bactericidal is plotted on the graph as a circle. For instance in this case, when the dose of penicillin was reduced to 16% M.L.C., the mixture was bactericidal when 25% M.L.C. of streptomycin was present.

In Table I are presented the results of similar studies on three strains of staphylococci and one strain of streptococci.

TABLE I.
Bactericidal Combinations of Penicillin and Streptomycin for Various Pure Cultures.

| Organism | % M.L.C.* Streptomycin | % M.L.C.* Penicillin | Sum |
|---|---|---|---|
| Hemolytic streptococcus C-203 MV | 5.6 | 50.7 | 56.3 |
| | 10.0 | 25.3 | 35.3 |
| | 14.3 | 23.0 | 37.3 |
| | 28.7 | 15.3 | 44.0 |
| | 43.4 | 5.0 | 48.4 |
| | 56.5 | 2.5 | 59.0 |
| *Staphylococcus aureus* No. 209 | 11.0 | 76.0 | 87.0 |
| | 13.0 | 61.0 | 74.0 |
| | 14.0 | 50.0 | 64.0 |
| | 28.0 | 38.0 | 66.0 |
| | 43.0 | 25.0 | 68.0 |
| | 56.0 | 12.0 | 68.0 |
| *Staphylococcus aureus* Oxford H | 15.3 | 50.0 | 65.3 |
| | 20.0 | 33.0 | 53.0 |
| | 25.3 | 16.0 | 41.3 |
| | 50.7 | 10.0 | 60.7 |
| *Staphylococcus aureus* | 10.0 | 76.1 | 86.1 |
| | 15.3 | 50.7 | 66.0 |
| | 25.3 | 20.0 | 45.3 |
| | 76.1 | 10.0 | 86.1 |
| *Staphylococcus aureus* SM | 10.0 | 76.1 | 86.1 |
| | 15.3 | 25.3 | 40.6 |
| | 25.3 | 20.0 | 45.0 |
| | 50.7 | 15.3 | 66.0 |
| *Staphylococcus aureus* SM | 10.0 | 50.7 | 60.7 |
| | 15.3 | 25.3 | 40.6 |
| | 25.3 | 20.0 | 45.3 |
| | 50.7 | 15.3 | 66.0 |
| | 76.1 | 10.0 | 86.1 |

* M.L.C. Minimum Lethal Concentration of each antibiotic alone for the particular organism determined to within 25% (*i.e.*, the organisms are known to grow at 75% M.L.C. or higher.)

*Summary.* Under the experimental conditions used, definite synergism was noted in the bactericidal action of penicillin and streptomycin upon staphylococci and streptococci *in vitro*.

16761 P

## A Complement Fixation Test for Dengue.*

ALBERT B. SABIN AND IRVING YOUNG.†
*From the Children's Hospital Research Foundation, Department of Pediatrics, University of Cincinnati College of Medicine.*

Studies carried out by one of us (A. B. S.) since 1944 have established the fact that several immunologically distinct but related types of virus are responsible for the clinically typical and atypical forms of dengue.[1] Since the adaptation of dengue virus to mice,[2] Sabin and Schlesinger found that the neutralizing antibodies which developed following infection were so highly type-specific, that by means of the neutralization test[3] in mice one

could not diagnose infections caused by heterologous immunological types of virus which have not as yet been propagated successfully in mice. Continuous serial, intracerebral passage in mice has increased the titer of our Hawaii strain of virus from $10^{-1}$ to about $10^{-5}$ or $10^{-6}$, and following intracerebral passage in 3- to 4-day-old mice (a procedure suggested by Dr. Gordon Meiklejohn) titers of $10^{-7}$ to $10^{-8}$ could be achieved. Satisfactory complement-fixing (C-F) antigens could be prepared from the brains of mice which were either 3 to 7 days old or approximately 14 days old at the time of inoculation with virus of highest titer ($0.03 \times 10^{-7}$-$0.03 \times 10^{-8}$) which could be obtained only from the younger suckling mice. Comparative tests on aliquot portions of 20% aqueous extracts of the infected mouse brains revealed that antigen prepared by benzene extraction of the lyophilized material[4,5] was no more potent as regards the number of antigenic units or the titer obtained with dengue antisera than that prepared only by centrifugation at 13,000 r.p.m. in the cold (Type SS-1 Swedish angle centrifuge—Sorvall), and both were equally effective in increasing the titer of complement. The 20% mouse brain suspension prepared by inoculation of 3- to 7-day-old mice contained 16 to 32 units of antigen per 0.25 ml as compared with 4 units (with two exceptions when it was only 1 unit and one when it was 8 units) for the similar suspension prepared in a similar manner from approximately 14-day-old mice. Almost all the work to be reported was carried out with benzene extracted antigens (containing 4 units), which, however, also had to be centrifuged at 13,000 r.p.m. for 1 hour to remove all the anticomplementary properties. The tests were set up in the usual manner, using 2 exact units of complement, and incubation for 16 to 20 hours in a refrigerator at about 5°C. The titers represent the highest original dilution of a serum, *i.e.*, before addition to the mixture, which yielded at least 2-plus fixation. Similarly prepared extracts from normal mouse brains were used as controls in all the tests.

*Results.* (1) Human volunteers, rhesus monkeys, and chimpanzees[6] inoculated with the homologous Hawaii strain of the *human* virus, or with immunologically identical *human* strains, developed C-F antibodies which reached peak titers of 1:64 to 1:256 within 2 to 6 weeks. These antibodies have been found to persist in high titer for many months, and in human volunteers, residing in dengue-free regions of the U. S. A., titers of 1:4 to 1:128 have been found 38 months after infection, and titers of 1:4 to 1:16 as long as 52 months after infection, the longest period tested.

(2) Human volunteers inoculated with immunologically distinct strains of *human* dengue virus, who developed little or no neutralizing antibodies for the Hawaii virus,[1] nevertheless, developed C-F antibodies in titers no higher than 1:16; however, 6 months after inoculation these antibodies were not demonstrable in serum dilutions of 1:4 in some of the individuals tested.

(3) Rhesus monkeys inoculated with immunologically distinct strains of *human* dengue virus, which developed little or no neutralizing antibodies for the Hawaii virus, nevertheless developed C-F antibodies in titers as high as 1:512; however, the fixation was not complete (*i.e.*, only 2- to 3-plus) even in the lowest dilutions of such sera, suggesting that only about 1 unit of the serologically related component was present in the antigens used. Furthermore, in some of the monkeys tested,

* This investigation was aided by the Commission on Virus and Rickettsial Diseases, Army Epidemiological Board, Office of The Surgeon General, Department of The Army.

† Lt., Medical Corps, A.U.S., on active duty.

1 Sabin, A. B., in Viral and Rickettsial Infections of Man, edited by T. M. Rivers, J. B. Lippincott Co., Philadelphia, 1948.

2 Sabin, A. B., and Schlesinger, R. W., *Science*, 1945, **101**, 640.

3 Sabin, A. B., Diagnostic Procedures for Virus and Rickettsial Diseases, Am. Pub. Health Assn., 1948, 289-293.

4 DeBoer, C. J., and Cox, H. R., *J. Immunol.*, 1947, **55**, 193.

5 Espana, C., and Hammon, W. McD., *J. Immunol.*, 1948, **59**, 31.

6 Paul, J. R., Melnick, J. L., and Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 193.

thus far, these antibodies disappeared as early as 6 to 10 weeks after inoculation.

(4) Rhesus monkeys inoculated intracerebrally with the homologous *mouse-adapted* Hawaii virus, developed C-F antibodies in titers as low as 1:16 which in some monkeys disappeared as early as 6 weeks after inoculation, although neutralizing antibodies in high titer persisted for many months.

(5) Human volunteers inoculated with dengue vaccine, consisting of the living mouse-adapted Hawaii virus, who developed neutralizing antibodies and resisted infection with the unmodified, human virus[1,7] *failed* to develop C-F antibodies.

(6) Sera obtained from individuals 2 years after a natural attack of dengue in Hawaii or Japan, which neutralized the Hawaii virus in high titer, also fixed complement in titers of 1:16 to 1:64. Positive results also have been obtained with sera of individuals who had dengue in Singapore, Java, and New Guinea.

(7) Sera collected from American marines, 11 months after a single, primary natural attack of dengue on Guam, gave positive C-F tests in titers of 1:2 to 1:8 with the Hawaii antigen, although they had no significant neutralizing antibodies[7] for this virus.‡

(8) Human volunteers infected with phlebotomus (pappataci, sandfly) fever virus developed no C-F antibodies for the dengue antigen.

(9) Rhesus monkeys which had no C-F antibodies for dengue 7 weeks after inoculation with the French neurotropic yellow fever virus, developed such antibodies in titers of 1:4 to 1:32, 8 to 10 days after a large booster dose of either the Asibi viscerotropic or French neurotropic strains of yellow fever virus.§ These results indicate an antigenic, if not an immunogenic,[1] relationship between the viruses of yellow fever and dengue.

[7] Sabin, A. B., and Schlesinger, R. W., unpublished studies.

‡ We are indebted to Drs. R. E. Shope and Horace Hodes, at the time serving with the U. S. Naval Medical Research Unit No. 2, for obtaining, lyophilizing and forwarding these sera in 1945.

§ We are indebted to Dr. Max Theiler, of the International Health Division of the Rockefeller Foundation, for the sera from these monkeys.

16762

## Influence of Choline, Cystine and Methionine on Toxic Effects of Pyridine and Certain Related Compounds.

ROLAND A. COULSON AND FRED G. BRAZDA.
(With the technical assistance of Lucy C. Gremillion.)
*From the Department of Biochemistry, Louisiana State University School of Medicine, New Orleans, La.*

In a study of the pharmacological effects produced by the ingestion of various pyridine derivatives by the rat[1,2] pyridine and coramine (nikethamide) were found to be the most active from the standpoint of the production of marked changes in liver size and composition. Coramine produced a great increase in liver weight without causing very much damage to the liver or modifying its composition. Pyridine has proved to be somewhat more toxic than many of its derivatives[1,3] and it must be included in the list of substances known to produce severe liver and kidney damage.[4,5] Methionine and cystine have been shown to have some liver protective

[1] Coulson, R. A., and Brazda, F. G., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 1.

[2] Brazda, F. G., and Coulson, R. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 37.

[3] Brazda, F. G., and Coulson, R. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 19.

[4] Baxter, J. H., *Proc. Am. Fed. Clin. Research*, 1945, **2**, 77.

[5] Baxter, J. H., *Am. J. Path.*, 1948, **24**, 503.

action when included in the diet of rats receiving pyridine.[4,6]

This report deals with the quantitative effects of the daily ingestion of pyridine or several heterocyclic compounds belonging to the quinoline series. The influence of various liver protective agents is also reported.

*Experimental.* The experimental animals consisted of equal numbers of male and female rats from our stock colony. The rats were placed on the test or control diets a few days after weaning. The basal diet had the same composition as that used for the coramine studies.[2] This diet consisted of 25% casein, 55% starch, 15% cottonseed oil and 5% salts. Five hundred ml of boiling water were added to each kg of dry diet. Each rat was given one ml of brewer's yeast extract a day and 3 drops of Percomorph oil a week.

The livers and kidneys were removed, weighed, and a small section was saved for histological examination. The livers were dried *in vacuo* and fat and water contents were determined as described by Brazda and Coulson.[2] Every effort was made to obtain the livers and kidneys for analysis from rats that had become moribund and had succumbed to the effect of the more toxic diets. Results obtained from these animals were so nearly alike regardless of the length of time the animals were on the diet that the data of all the moribund rats in a given experiment were gathered together into one group. In order to eliminate the effects of postmortem changes in the composition of the liver the tables presented do not include the results from any animal that had been dead for over 10 minutes. Records were kept of the growth rate, the food consumption, and length of life of the experimental animals.

In all, about 1200 rats were placed on diets containing pyridine or quinoline (in the form of acetates) to which various supplements suspected of being liver protective were added. Each rat received a fresh lump of the experimental diet each day and any diet left over from the previous day was discarded. The animals were weighed at the beginning of the experiment and at the time of death or at the time they were killed for autopsy. In all cases the experiments were concluded at 28 days. The experiments were conducted at an average temperature of about 75°F. As the season progressed the animals were removed to a room where the temperature was maintained at 76°F and the relative humidity at 50%.

*Pyridine Series.* Each group contained 70 rats, one female and one male from each of 35 litters. As far as was possible each litter contributed rats to 3 experimental groups. Group I received a diet which contained 0.6% pyridine; group II 0.6% pyridine plus 0.5% choline; group III 0.6% pyridine plus 1% cystine; group IV 0.6% pyridine plus 1.2% methionine; group V 0.6% pyridine plus 0.6% beta picoline; and group VI 0.6% pyridine plus 1.0% nicotinamide. In spite of the unpleasant odor of the diet the rats ate it willingly. There was no great difference in either food consumption or growth rate in the first 4 groups. The addition of beta-picoline or nicotinamide greatly reduced food intake which in turn reduced the growth rate. Two other pyridine groups were set up to determine the reproducibility of the survival curves. These groups are not included in Table I since there was no significant variation in the shape or the absolute area contained within the curve and hence it was concluded that 70 rats were sufficient for testing the effect of pyridine on survival.

The results are summarized in Fig. 1. Probably the best index of the relative survival time is obtained by comparing the respective areas under the curves. Considering a control curve to have an area of 1000, then the pyridine curve has an area of 348, the pyridine plus choline 465, the pyridine plus cystine 706, and pyridine plus methionine 773. It is apparent that methionine and cystine are far more effective in prolonging the life of the experimental animals than choline in spite of the fact that choline is known to be a lipotropic compound whereas cystine is ineffective in this respect. These results are in agreement with those reported by Baxter.[6] The survival curves of the groups receiving supplements of beta-picoline or nicotinamide in addition to the pyridine are not included in

[6] Baxter, J. H., *J. Clin. Invest.*, (*Proc.*), 1946, **25**, 908.

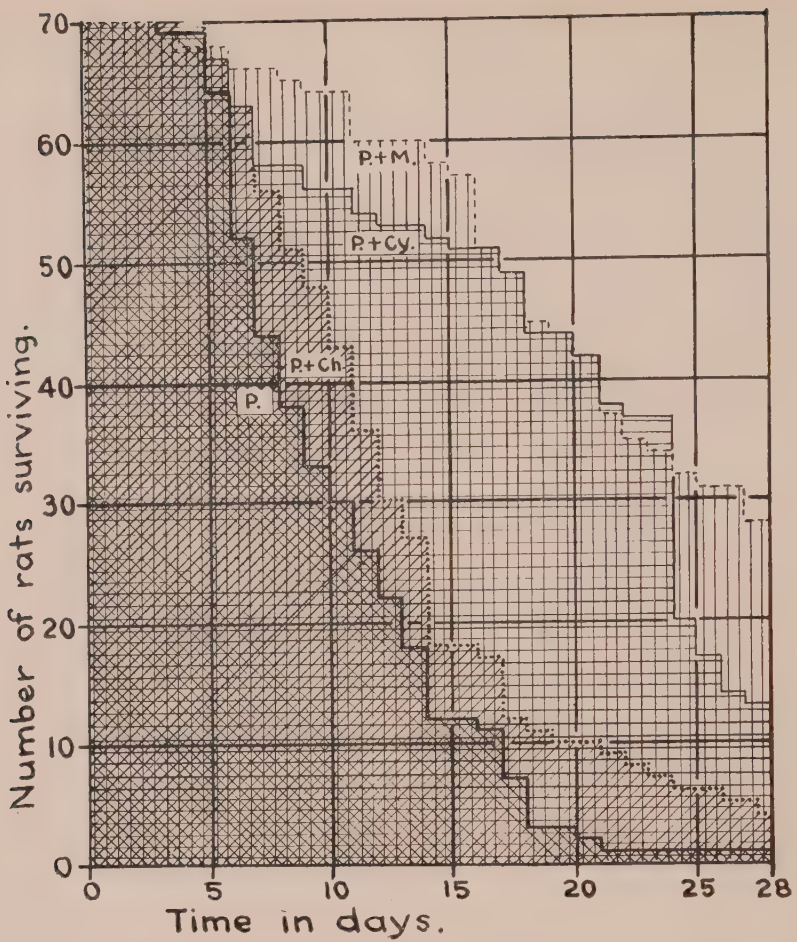



FIG. 1.

Survival of Rats on Pyridine Diets. P = 0.6% pyridine; P + Ch = 0.6% pyridine plus 0.5% choline; P + Cy = 0.6% pyridine plus 1.0% cystine; P + M = 0.6% pyridine plus 1.2% methionine.

Fig. 1. Both groups of animals ate much less and grew at a correspondingly lower rate which introduced the factor of food consumption. The area under the curves was greater than that found in the pyridine or pyridine and choline groups but much less than that found in the pyridine and cystine or pyridine and methionine groups. Paired feeding experiments are necessary to evaluate the influence of these two compounds since a decreased food intake means a correspondingly decreased intake of pyridine.

Table I presents the results of the liver analyses for the 6 groups of 70 rats each and for 10 rats which were fed piperidine, the reduced derivative of pyridine. The table may be summarized by stating that all of the rats in the pyridine experiments had marked liver enlargement. Without exception the moribund rats had livers which were larger than those of the sacrificed animals and which had a greatly decreased solid content. There are no significant differences in the growth rate of the animals receiving pyridine alone of those receiving additional supplements of choline or methionine. The cystine series grew at a rate somewhat below that of the others. Although the food intake and the growth rate paralleled each other rather closely both were subject to considerable individual variation.

The average fat contents of the pyridine

TABLE I.

Effect of Various Compounds on Size and Composition of Livers of Rats Receiving Pyridine.

| Group | No. rats | No. days | Liver wt as % body wt | | Liver fat, % dry wt | Liver % solids, avg | Gain in wt, g/day |
|---|---|---|---|---|---|---|---|
| | | | Avg | P.E.* | | | |
| 0.6% pyridine—*Sacrificed* | 11 | 5 | 6.21 | ±0.25 | 17.36 | 31.26 | +1.42 |
| | 10 | 9 | 6.39 | ±0.12 | 21.33 | 33.05 | +2.33 |
| 0.6% pyridine—*Moribund* | 12 | — | 8.96 | ±0.24 | 24.14 | 25.08 | — |
| 0.6% pyridine + 0.5% choline—*Sacrificed* | 10 | 5 | 5.84 | ±0.18 | 13.24 | 29.38 | +1.81 |
| | 13 | 9 | 5.79 | ±0.12 | 11.49 | 28.23 | +1.99 |
| | 2 | 28 | 5.57 | — | 11.76 | 31.03 | +2.15 |
| 0.6% pyridine + 0.5% choline—*Moribund* | 18 | — | 8.41 | ±0.17 | 12.60 | 22.91 | — |
| 0.6% pyridine + 1% cystine—*Sacrificed* | 12 | 28 | 9.75 | ±0.33 | 16.23 | 26.52 | +1.31 |
| 0.6% pyridine + 1% cystine—*Moribund* | 5 | — | 10.33 | ±0.31 | 13.03 | 24.53 | — |
| 0.6% pyridine + 1.2% methionine—*Sacrificed* | 11 | 5 | 5.89 | ±0.11 | 14.89 | 31.29 | +1.13 |
| | 12 | 9 | 7.03 | ±0.16 | 16.17 | 28.75 | +1.66 |
| | 28 | 28 | 6.50 | ±0.11 | 10.38 | 29.00 | +1.97 |
| 0.6% pyridine + 1.2% methionine—*Moribund* | 10 | — | 9.50 | ±0.13 | 12.14 | 22.91 | — |
| 0.6% pyridine + 0.6% beta picoline—*Sacrificed* | 9 | 28 | 8.77 | ±0.25 | 17.19 | 31.11 | +1.18 |
| 0.6% pyridine + 0.6% beta picoline—*Moribund* | 7 | — | 9.44 | ±0.37 | 21.02 | 24.69 | — |
| 0.6% pyridine + 1.0% nicotinamide—*Sacrificed* | 17 | 28 | 7.96 | ±0.31 | 14.78 | 28.70 | +0.57 |
| 0.6% pyridine + 1.0% nicotinamide—*Moribund* | 7 | — | 10.44 | ±0.24 | 16.47 | 25.38 | — |
| 0.6% piperidine | 10 | 28 | 4.93 | ±0.14 | 7.59 | 28.46 | +1.30 |
| Control | 10 | 5 | 4.98 | ±0.09 | 13.27 | 29.21 | +3.21 |
| | 10 | 9 | 4.78 | ±0.09 | 13.38 | 29.15 | +3.30 |
| | 10 | 28 | 5.18 | ±0.13 | 10.89 | 29.48 | +3.79 |

* P.E. $= 0.6745 \sqrt{\frac{\Sigma(v)^2}{n(n-1)}}$

TABLE II.
Effect of Injection of a Lethal Dose of Pyridine on the Water Content of the Rat Liver.

| Group | No. rats | Final wt avg | Liver wt as % body wt | | Liver fat, % dry wt | Liver % solids, avg |
|---|---|---|---|---|---|---|
| | | | Avg | P.E. | | |
| Experimental | 18 | 49.5 | 4.90 | ±0.11 | 8.66 | 25.40 |
| Controls | 18 | 57.6 | 4.78 | ±0.17 | 14.62 | 32.60 |

and pyridine plus cystine groups are slightly higher than those of the groups receiving the lipotropic factors choline or methionine. The high protein diet is instrumental in preventing an excessive accumulation of fat in the livers of all groups. Piperidine had no noticeable effect on the liver although it did decrease food intake and growth rate.

Nine males and 9 female rats whose average age was 25 days were injected subcutaneously with pyridine in amounts that were calculated to be 3 times the $LD_{50}$.[3] These animals died in from 1 to 4 hours and the livers were removed, analyzed, and a piece was saved for histological study. The results appear in Table II. No significant liver enlargement was noted although the livers did show an increase in the average water content. The solid content was still considerably above the solid content found for the moribund group in Table I. In general the picture presented by the group injected with lethal doses of pyridine is quite different from that of the rats which were fed pyridine. It is probable these animals died from respiratory paralysis rather than from liver failure.

*Quinoline Series.* The survival curves found in Fig. 2 were obtained in the same manner as those of the pyridine experiments. Quinoline was used at a dietary level of 1% which is equivalent to 0.6% pyridine. If the control curve is given an arbitrary value of 1000 the relative area under the methionine curve is 812, the cystine is 745, the choline is 648, and the quinoline alone is 535. The fact that the relative order of effectiveness of the liver protective compounds is the same as in the pyridine groups suggests that the same mechanism is involved in both experiments. Methionine is again somewhat superior to cystine. Since the addition of choline does prolong the life of the quinoline rats it is probable that the slight difference between the pyridine and pyridine and choline groups in Fig. I is significant.

The results of the liver analyses are presented in Table III. The livers are very greatly enlarged in all 4 groups, approaching one-seventh of the total body weight. Although none of the rats presented in this table were moribund, the values for the liver solid content of the quinoline and the quinoline and choline groups closely resemble the figures obtained from the moribund rats of the pyridine groups. Without resort to histological examination it is apparent that cystine and methionine retard or prevent the hydration of the livers.

In the pyridine series it was observed that neither methionine nor cystine had any beneficial effect on the rate of growth of the animals on the diet reported in this paper. Quinoline alone retards the growth rate seriously and the addition of choline does not prevent this retardation. However the inclusion of either cystine or methionine increases the growth rate of the animals in a dramatic fashion. In the entire series of rats receiving quinoline with or without choline not one rat gained weight over the 28-day period. Of the 99 rats receiving quinoline plus cystine or methionine which survived long enough to be autopsied only 2 failed to gain weight. The growth rate was commensurate with the food intake which means that although those rats which received cystine or methionine ingested more quinoline, they remained in better health and lived longer than those of the other two groups.

A number of animals that had become moribund after eating diets which contained quinoline were examined immediately after death. It was not possible to obtain enough of these for the results to be of statistical significance. Those animals that died in the first few days had eaten so little of the diet that starvation

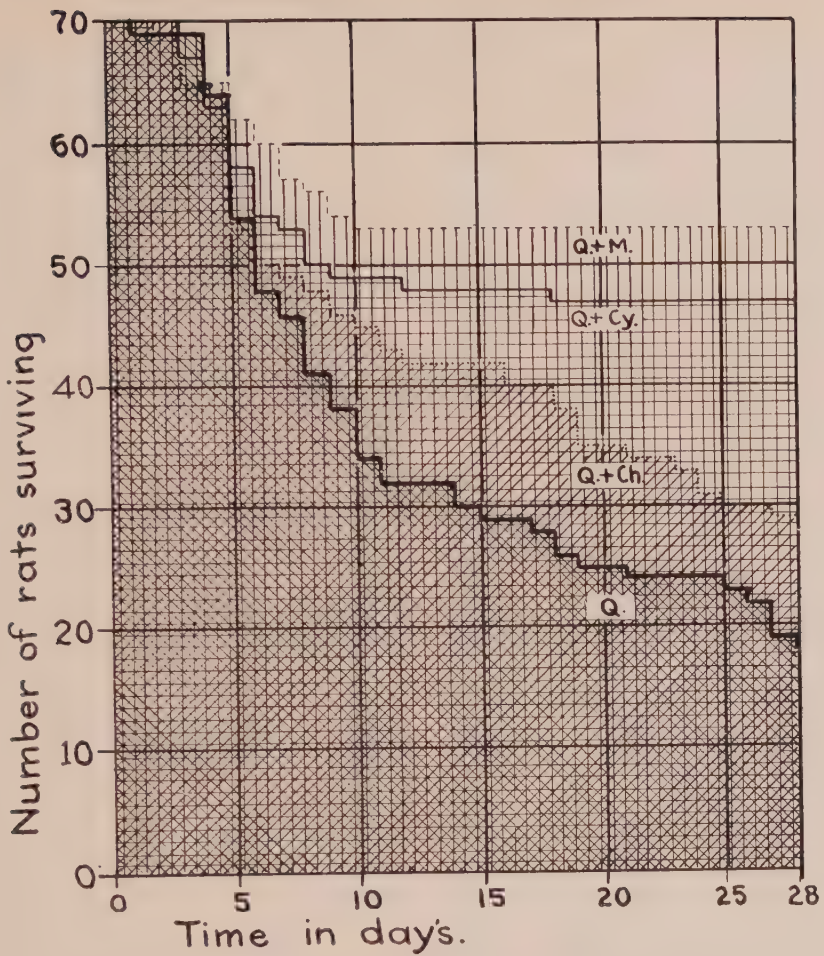



FIG. 2.
Survival of Rats on Quinoline Diets. Q = 1.0% quinoline; Q + Ch = 1.0% quinoline plus 0.5% choline; Q + Cy = 1.0% quinoline plus 1.0% cystine; Q + M = 1.0% quinoline plus 1.2% methionine.

probably contributed to a large extent to their death. In spite of this, gravimetric analyses of the livers of these few moribund rats gave results which were similar to those from the moribund animals of the pyridine groups. The very great increase in the liver weight of the sacrificed rats in the quinoline and quinoline and choline groups reported in Table III indicates that all of these experimental animals ate enough of the diet to produce liver damage.

On the basis of the theory that the tertiary nitrogen of quinoline is responsible for its action on the liver, quinoline was methylated to quinoline methochloride and this derivative was fed to 5 male and 5 female rats. The methochloride proved to be extremely toxic at a dietary level of 1.39% which is equivalent to a 1% quinoline level. Seven of the 10 rats died in less than a week; the other 3 were arbitrarily killed at 7 days and examined. The livers were normal in every respect and the experiments with this compound were not continued.

Isoquinoline at a level of 1% of the diet was also very toxic. Of the 20 rats that were fed this substance only 7 remained alive at 5 days. These 7 had livers which were hydrated to some extent although the degree of the liver enlargement was not marked. Isoquinoline methochloride was as toxic as isoquinoline but it produced no noticeable effect on liver size

TABLE III.
Effect of Choline, Cystine, or Methionine on Livers of Rats Receiving Quinoline.

| Group | No. rats | No. days | Liver wt as % body wt | | Liver fat, % dry wt | Liver % solids, avg | Gain in wt, g/day |
|---|---|---|---|---|---|---|---|
| | | | Avg | P.E. | | | |
| 1.0% quinoline | 18 | 28 | 11.27 | ±0.24 | 11.62 | 24.10 | —0.40 |
| 1.0% quinoline + 0.5% choline | 28 | 28 | 14.69 | ±0.10 | 18.21 | 25.92 | —0.63 |
| 1.0% quinoline + 1.0% cystine | 47 | 28 | 14.49 | ±0.13 | 14.49 | 29.69 | +0.39 |
| 1.0% quinoline + 1.2% methionine | 52 | 28 | 11.09 | ±0.16 | 11.15 | 28.55 | +0.36 |

or composition.

*Histological Appearance of the Livers.* The ingestion of pyridine causes the production of cirrhosis or more commonly necrosis or both in the young rat. This is in agreement with results recently reported by Baxter.[5] On histological examination the livers of the moribund rats appeared to be hydrated and showed many small irregular vacuoles which were similar to those described by Trowell.[7] Gravimetric analyses of the whole livers, which usually reveal more information than does microscopic examination of small sections, clearly showed not only the presence of extra water but also the magnitude of its increase. In animals that died during the experiments with pyridine the histological picture of the livers was the same regardless of the presence or absence of a liver-protective supplement. The apparently healthy rats that were killed arbitrarily at different time intervals had livers which showed little damage. It would appear, therefore, that the development of severe necrosis following the ingestion of pyridine requires a relatively short period of time. Damage to the kidney is roughly but not absolutely proportional to the damage to the liver. Although many cases of kidney damage were seen few of these were severe enough to be considered as the primary cause of death and it was concluded that most of those animals that died from the ingestion of pyridine died from liver failure. Further evidence that liver failure was the cause of death may be obtained from consideration of the fact that all of the moribund rats had livers which were very severely damaged as shown by gravimetric analysis. The injection of lethal doses of pyridine into young rats had no effect on the histological appearance of the livers.

The animals of the quinoline series had livers which were more nearly normal than those in the pyridine groups as far as the microscopic picture was concerned. Although the cells were swollen and some small areas of necrosis were seen the overall appearance of the liver was not suggestive of marked damage. The percentage liver weights were from 2 to 2½ times the control weights, yet the extent and nature of the enlargement was not apparent from the slides. It is by no means certain that rats which died while receiving quinoline in the diet died of liver failure.

*Discussion.* The survival curves are useful in determining the toxic effects of compounds of the pyridine or quinoline nucleus and the influence of certain agents that reduce the toxicity of these substances. The sulfur containing aminoacids, methionine and cystine, are superior to choline in preventing liver damage. The fact that cystine is a good protective agent suggests that transmethylation is probably not involved in this protection since there is no evidence that cystine can contribute anything to methylation of the ring nitrogen as a means of detoxication. Although pyridine and quinoline may be methylated by the rat there is still no conclusive evidence that this is the case.[1] It is possible that the sulfur-containing compounds are involved in the detoxication of quinoline and pyridine in a manner as yet unknown.

Trowell[7] has reported that intact rats whose livers have been subjected to procedures designed to produce anoxia without reduction

[7] Trowell, O. A., *J. Physiol.*, 1946, **105**, 268.

of the intrasinusoidal pressure develop watery vacuoles in this organ. Inasmuch as the liver in a rat dying from effects of pyridine becomes hydrated, especially during the last hours of life, it seems logical to assume that this is due basically to a decrease in the rate of oxidation in the cells.

*Summary.* Pyridine alone or pyridine supplemented with either choline, cystine, methionine, nicotinamide or beta-picoline was fed to young rats on a high protein diet. Livers from rats dying from the effects of pyridine are enlarged and always exhibit a marked increase in water content. Livers from apparently healthy animals which have been on a pyridine diet show a nearly normal water content and are enlarged. Cystine and methionine afford protection against the effects of pyridine. The feeding of quinoline alone or quinoline supplemented with either choline, cystine, or methionine produced enlarged livers. Cystine and methionine increased survival time. Choline showed some protective effect. Cystine or methionine prevented the accumulation of water in the livers of rats fed quinoline. Choline had no effect.

16763

## Quantitative Detection of Minute Concentrations of Digitoxin.*

RENÉ BINE, JR. AND MEYER FRIEDMAN.

*From Harold Brunn Institute, Mount Zion Hospital, San Francisco, Calif.*

The detection of a digitalis glycoside, Lanatoside C, in various media by means of the embryonic duck heart preparation has been reported in previous studies.[1-3] It was found in these same studies that not only could extremely minute amounts of the glycoside (less than 0.1 μg per cc) be detected by this preparation, but also quantitative estimations of the actual amount of glycoside in Tyrode's solution or serum could be determined.

However, the sensitivity of the embryonic duck heart to the digitalis glucoside, digitoxin, had not been determined. Accordingly, studies were made of the effect of various concentrations of digitoxin upon the duck heart immersed in (1) Tyrode's solution, (2) rat serum, and (3) human serum. The method of quantitation employed was the same as that described previously.[1,3] It was found that the embryonic duck heart was not only extraordinarily sensitive to minute quantities of digitoxin but also offered a means whereby the concentration of digitoxin could be assessed in a quantitative fashion.

*Results.* As Table I demonstrates, the embryonic heart preparation was able to detect the presence of 0.005 μg of digitoxin in one cc of Tyrode's solution. Moreover, with increasing concentrations of the drug, there was a progressive reduction in the time taken for the occurrence of the digitalis effect.

The action of digitoxin in rat serum was found to be much less effective than in Tyrode's solution. Thus (Table I) only quantities of 0.2 μg or more of digitoxin per cc could be detected. Human serum was even more inhibitory (Table I) in that only quantities of 0.60 μg or more of digitoxin per cc could be detected. This action of serum upon the action of digitoxin also has been demonstrated by previous observers.[4,5] Lanatoside C, how-

* Aided by grants from the Life Insurance Medical Research Fund, The Public Health Service and The Sandoz Chemical Works.

1 Friedman, M., and Bine, R., Jr., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 162.

2 Friedman, M., and Bine, R., Jr., *Am. J. Med. Sci.*, 1947, **214**, 633.

3 Bine, R., Jr., and Friedman, M., *Am. J. Med. Sci.*, 1948, **216**, 534.

4 Fawaz, G., and Farah, A., *J. Pharm. and Exp. Therap.*, 1944, **80**, 193.

5 Suter, E., *Helvet. Physiol. et Pharmacol. Acta*, 1944, **2**, 2.

TABLE I.
The Detection of Digitoxin in (1) Tyrode's Solution, (2) Rat Serum, and (3) Human Serum by the Embryonic Duck Heart Preparation.

| | Tyrode's solution | | | Rat serum | | | Human serum | | |
|---|---|---|---|---|---|---|---|---|---|
| Conc. of digitoxin ($\mu$g/cc) | Avg. time "dig. effect" (min.) | Stand. error of mean (min.) | No. hts. | Avg. time "dig. effect" (min.) | Stand. error of mean (min.) | No. hts. | Avg. time "dig. effect" (min.) | Stand. error of mean (min.) | No. hts. |
| .001 | ND | | 10 | ND | | 12 | ND | | 10 |
| .005 | 48 | 3.0 | 15 | ND | | 10 | ND | | 10 |
| .010 | 41 | 1.6 | 21 | ND | | 10 | ND | | 10 |
| .05 | 23 | 1.5 | 33 | ND | | 10 | ND | | 10 |
| .10 | 12 | 0.8 | 20 | ND | | 10 | ND | | 10 |
| .20 | — | — | — | 62 | 1.3 | 18 | ND | | 15 |
| .40 | — | — | — | 38 | 1.7 | 18 | ND | | 15 |
| .60 | 6 | 0.2 | 20 | 24 | 0.5 | 36 | 49 | 2.0 | 23 |
| .80 | — | — | — | 21 | 0.8 | 20 | 34 | 1.6 | 24 |
| 1.00 | 4 | 0.2 | 29 | 11 | 0.4 | 22 | 21 | 1.1 | 28 |

ND indicates that no glycoside could be detected by occurrence of "digitalis effect" in embryonic heart preparation.

ever, was not found[3] to be inhibited similarly by serum.

*Conclusions.* 1. Quantitative assay of extremely minute amounts of digitoxin in Tyrode's solution and in rat and human serum was found to be possible by means of the embryonic duck heart preparation.

## 16764

## Peculiar Enlargement of Eyeballs in Chicks Caused by Feeding a High Level of Glycine.*

A. C. GROSCHKE,† J. O. ANDERSON, AND G. M. BRIGGS.‡
*From the Department of Poultry Husbandry, University of Maryland, College Park, Md.*

Previous reports[1,2] from this laboratory have shown that glycine is highly "pellagragenic" when fed to chicks receiving a nicotinic acid-low diet. On the other hand, the chick tolerated unusually high levels of glycine (4 to 6%), provided a sufficient amount of nicotinic acid was contained in the diet.[2] The latter observation was somewhat surprising for it had been reported earlier that more than 2% of glycine was toxic to the young chicken[3] and that 4 g of glycine per day were harmful to hens.[4]

Because of this discrepancy, an investigation was undertaken to determine the minimum toxic level of glycine and the extent to which this level could be influenced by nicotinic acid feeding. The present report describes a peculiar overgrowth of the eyeballs and other conditions observed in chicks resulting from feeding a highly purified diet con-

* Scientific Paper No. A202. Contribution No. 2119 of the Maryland Agricultural Experiment Station (Department of Poultry Husbandry).

† Now at Michigan State College, East Lansing, Mich.

‡ Now at University of Minnesota, St. Paul, Minn.

1 Briggs, G. M., Groschke, A. C., and Lillie, R. J., *J. Nutrition*, 1946, **32**, 659.

2 Groschke, A. C., and Briggs, G. M., *J. Biol. Chem.*, 1946, **165**, 739.

3 Almquist, H. J., Stokstad, E. L. R., Mecchi, E., and Manning, P. D. V., *J. Biol. Chem.*, 1940, **134**, 213.

4 Patton, A. R., *Poultry Sci.*, 1939, **18**, 31.

TABLE I.
Composition of Basal Diet 113GN.

| | % |
|---|---|
| Cerelose | 71.4 |
| Casein (crude) | 18.0 |
| Mineral mixture 1M | 6.0 |
| Soybean oil | 4.0 |
| *dl*-Methionine | 0.3 |
| Choline chloride | 0.2 |
| *i*-Inositol | 0.1 |
| Vitamins (mg/100 g). | |
| Thiamine HCl | 0.4 |
| Riboflavin | 0.8 |
| Ca pantothenate | 2.0 |
| Pyridoxine HCl | 0.6 |
| Biotin | 0.02 |
| Pteroylglutamic acid | 0.2 |
| Para-aminobenzoic acid | 0.2 |
| 2-Methyl-1,4-naphthoquinone | 0.1 |
| Alpha-tocopherol | 0.5 |
| Vit. A and $D_3$, 1200 U.S.P. and 170 A.O.A.C. units, respectively, by dropper weekly. | |

taining 8% of glycine.

*Experimental.* Day-old New Hampshire chicks of mixed sexes were used. The chicks were distributed according to weight, confined in electrically heated wooden cages with wire screen floors and given feed and water *ad libitum*. Weighings and other observations were made weekly. All substitutions in the basal diet 113GN were made at the expense of cerelose (Table I).

*Results.* Table II summarizes the results of feeding 8% of glycine to chicks in combination with various levels of nicotinic acid. It is quite evident that glycine at this level inhibits growth and that the growth depression is not overcome by feeding nicotinic acid in amounts as high as 100 mg per 100 g of diet. A few cases of perosis occurred in the groups receiving glycine and feathering was inferior. No "chick black-tongue" was seen, as might be expected with the high level of glycine fed.

During the first week of the experiment the chicks in the 3 groups receiving glycine exhibited periods of extreme prostration accompanied by tremors. This condition seemed to be especially severe after eating. Sometimes the chicks would become so comatose they would appear to be at the point of death at which time they would gradually rally. In spite of these symptoms, constant weight gains were made.

After the first week the chicks appeared to outgrow the tendency toward prostration, but slight tremors existed in the birds throughout the whole experiment. They seemed to be constantly fatigued and would stretch their wings and legs at frequent intervals.

At about the third week a peculiar puffiness about the eyes was noticed. By the fourth week it was evident that either the eyeballs were becoming enlarged or that an edemic condition existed in the optic vesicle which was causing the eye to be forced out, as occurs in exophthalmic goiter in the guinea pig. The eye condition at this point was severe enough to interfere with the normal movements of the nictitating membrane.

At the end of 4 weeks, 3 typical chicks from each group receiving glycine were selected and the total of 9 chicks were given the diet containing 8% of glycine and 10 mg of nicotinic acid per 100 g of diet. They were continued on experiment to 7 weeks of age along with the 6 control chicks. From the fourth week to the seventh week, the "popped" eye condition became worse. From about the fifth week on, the eyes were so bulged that normal vision was impaired to the point where it was evident that the eyes could not be focused on objects close at hand. Consequently, the feed hoppers had to be kept quite full so that the chicks could eat properly.

At 7 weeks of age all the chicks were killed by severing the jugular vein. All had been fasted for a period of 6 hours prior to killing. The heart, liver with bile sac, spleen, thyroids, and one eye from each chick were removed and weighed immediately. All adhering muscular tissue was cut away from the eyes before they were weighed. In Table III the average values obtained for the various organs are given expressed in terms of percent of body weight. The data show that on this basis the values of the 2 groups for the thyroids, heart, liver plus bile sac, and spleen were not appreciably different. However, a marked difference existed in the weight of the eyeballs. The eyes from the chicks which received 8% of glycine were almost twice as great on a percent-of-body-weight

TABLE II.
Effect of 8% of Glycine on Chicks Receiving Various Levels of Nicotinic Acid.

| Group No. | Supplement of basal diet 113GN containing 5% gelatin | No. chicks | No. dead | Avg wt 4 wk, g | No. perosis | Feed efficiency* |
|---|---|---|---|---|---|---|
| 1 | 10 mg % nicotinic acid | 6 | 0 | 235 | 0 | .510 |
| 2 | 10 " " " " + 8% glycine | 6 | 1 | 157 | 2 | .501 |
| 3 | 50 " " " " + " " | 6 | 1 | 152 | 1 | .551 |
| 4 | 100 " " " " + " " | 6 | 1 | 158 | 1 | .504 |

* $\frac{\text{Total weight gained}}{\text{Total feed consumed}}$ = feed efficiency.

TABLE III.
Effect of 8% of Glycine in the Diet of Chicks on the Size of Various Organs of the Body.

| Diet | No. chicks | Avg body wt 7 wk, g | % of body weight (avg) Eye* | Thyroid† | Heart | Liver and bile sac | Spleen |
|---|---|---|---|---|---|---|---|
| A | 6 | 532 | 0.343 (0.289-0.396) | 0.008 | 0.503 | 2.776 | 0.210 |
| B | 9 | 399 | 0.638 (0.565-0.762) | 0.007 | 0.537 | 2.893 | 0.232 |

Diet A. 113GN + 5% gelatin, 10 mg % nicotinic acid (control group).
Diet B. 113GN + 5% gelatin, 10 mg % nicotinic acid, 8% glycine.
* Values given are for one eye.
† Values given are for both thyroids.

basis as were the eyes from the controls. This striking eye condition may be seen in Fig. 1 which shows the difference in size of 2 excised eyeballs, one from a control chick and the other from a chick which received 8% of glycine.

The production of an apparent overgrowth of the eyeballs seems to be related to the form in which the amino acid is fed. The same basal diet supplemented with 10% of gelatin, 25% of bone ossein, and 10 mg of nicotinic acid per 100 g did not cause an enlargement of the eyeballs, tremors, or marked growth depression, although this diet contained approximately 8% of glycine but in the peptide form. (Bone ossein was used in place of gelatin because the diet tended to stick to the beaks of chicks when it contained more than 10% of gelatin. It is assumed that bone ossein and gelatin are equally digestible because both depressed growth in about the same order of magnitude when fed to chicks receiving nicotinic acid-low diets. No explanation for the protective action of protein is offered.)

Furthermore, the effect of free glycine under the conditions of these experiments is confined to the growing chick. In experiments with laying hens, 4, 8, and 12% of glycine in a semi-purified diet containing 50 mg of nicotinic acid per 100 g did not cause any eye abnormalities during successive feeding periods of 5, 3, and 4 weeks, respectively. Feed consumption and egg production were only slightly lowered during the latter 4-week treatment.

The underlying physiological cause of the observed biological anomaly is as yet unknown. Whether the enlargement of the eyeballs is the result of actual tissue growth, or increased optic fluid caused by disturbed osmotic relationships, or other conditions must be determined through additional experimentation. The fact that the condition was observed in young growing chickens and not in laying hens would lend support to the belief that the enlargement is the result of actual overgrowth of tissue and not to increased ocular fluid caused by osmotic disturbances. Whether or not other amino acids will cause this same condition has not been studied.

*Summary.* Growth depression and tremors were observed in chicks fed 8% of glycine as part of a highly purified diet. The depressed growth rate was not overcome by feeding unusually high levels of nicotinic acid. In addi-

FIG. 1.
Contrast in size of eyeballs. The eyeball on the left is from a 7-week-old chick which received a diet containing 8% of glycine; its body weight was 395 g. The eyeball on the right is from a normal 7-week-old chick; its body weight was 574 g.

tion, a peculiar enlargement of the eyeballs developed which interfered with normal vision and functioning of the nictitating membrane. Eight percent of glycine as the peptide produced no such effect. The action of glycine appeared to be confined to the growing chicken. The feeding of 12% of glycine to hens over an extended period produced no apparent ocular abnormalities.

We are indebted to Merck & Co., Rahway, N. J., for crystalline vitamins; Wilson and Co., Inc., Chicago, Ill., for 2X gelatin; Allied Mills, Inc., Portsmouth, Va., for soybean oil; Abbott Laboratories, North Chicago, Ill., for haliver oil; U. S. Industrial Chemicals, Inc., Baltimore, Md., for *DL*-methionine; and to Lederle Laboratories, Pearl River, N. Y., for pteroylglutamic acid.

16765

## Effect of Dicumarol on Ac-Globulin and Prothrombin Activity.*

JOHN L. FAHEY, JOHN H. OLWIN, AND ARNOLD G. WARE.
(Introduced by Walter H. Seegers.)

*From the Department of Physiology and Pharmacology, Wayne University College of Medicine, Detroit, and the Department of Surgery, The Presbyterian Hospital of the City of Chicago.*

The administration of dicumarol to prevent the occurrence or extension of intravascular thromboses has given encouraging results.[1-3] The rationale of this treatment is based upon the ability of dicumarol to lower the prothrombin concentration in the circulating plasma.[3] Dicumarol has been thought to act principally upon the prothrombin portion of the coagulation mechanism and thus to depress the clotting activity of whole blood. It is now apparent that another factor, Ac-globulin, warrants study in connection with dicumarol therapy. Ac-globulin[4] is a plasma protein normally found in the circulating plasma. It acts to assure normal physiological conversion of prothrombin to thrombin during the process of blood coagulation. A deficiency of this factor impairs thrombin formation and a bleeding tendency may develop.[5]

The existence of Ac-globulin was unknown until recently, and previous reports on prothrombin measurements by both the one- and two-stage tests have not considered the possible role of this factor. With accurate and specific methods now available, a study was undertaken of both prothrombin and Ac-globulin levels as they are influenced by dicumarol administration.

*Methods.* Blood was obtained by venipuncture. In order to reduce contamination by tissue fluids the initial 3 cc of blood drawn into the syringe were discarded. The sample to be analyzed was received into a second syringe containing the anticoagulant. In this

* Aided by grants from the United States Public Health Service and the Ortho S. A. Sprague Memorial Institute Fund.

1 Olwin, J. H., Josiah Macy, Jr., Conference on Blood Clotting and Allied Problems, N. Y., 1948.

2 Allen, E. V., Hines, E. A., Kvale, W. F., and Barker, N. W., *Ann. Int. Med.*, 1947, **27**, 371.

3 Link, K. P., *Harvey Lectures*, 1943-44, 162.

4 Ware, A. G., and Seegers, W. H., *J. Biol. Chem.*, 1948, **172**, 699.

5 Owren, P. A., *Lancet*, 1947, **252**, 446.

manner 9 volumes of blood were added to 1 volume of 3.2% sodium citrate. After centrifugation at 3,000 rpm for 30 minutes the plasma was carefully removed from the cellular elements and frozen at –20°C until analyzed. Quantitative prothrombin determinations were carried out by means of a modified 2-stage test in which Ac-globulin is supplied.[6] By adding an excess of accelerator in this modification, maximum conversion of prothrombin to thrombin is assured under the conditions specified in the 2-stage prothrombin analysis.[7]

Ac-globulin was measured by a method in which the concentrations of prothrombin, thromboplastin, and other known variables are controlled with the result that the rate of thrombin formation is dependent upon the amount of Ac-globulin present.[4] Because this test for Ac-globulin is a measure of a reaction rate (prothrombin to thrombin) and quite sensitive to minor changes in the reacting medium, all determinations were related to a control plasma of the same species. The normal control plasmas were carefully collected and frozen at –20°C until they were measured at the same time as the unknown samples.

*Experimental.* 1. *Canine species.* Seven normal healthy dogs were selected and the normal levels of plasma prothrombin and Ac-globulin determined. On successive days dicumarol was administered orally either as 2 doses of 14 mg per kg of body weight or as 4 doses of 4 mg per kg. Samples of blood for analysis were obtained regularly either from the saphenous or antecubital vein.

The results of these experiments indicate a sharp drop in plasma prothrombin which is maintained for a period of about a week. Following this a gradual restoration of prothrombin activity takes place. In addition, there was generally an initial decrease in Ac-globulin activity upon dicumarolization, though the magnitude of fall was much less than that of the prothrombin. This reduction

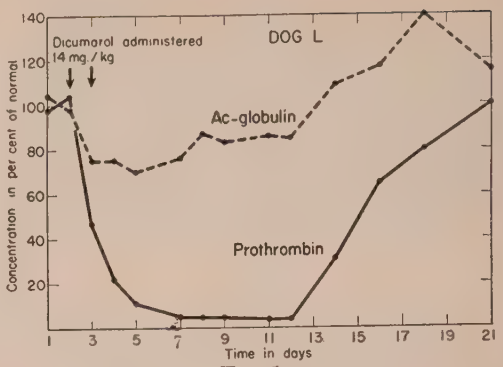



FIG. 1.

Changes in plasma Ac-globulin and prothrombin concentrations from dicumarol administration to a dog.

in Ac-globulin is only moderate, rarely falling under 65% of normal, and is in contrast to the severe fall in the prothrombin concentration. Even with the larger doses of dicumarol the Ac-globulin level did not fall below 50% of normal as compared to prothrombin titers reduced to as low as 1% of normal. In Fig. 1 are illustrated the results of a typical experiment employing a 9.0 kg female dog.

Considerable individual variation in the depth and duration of the response of Ac-globulin was observed. The drop generally began on the day following the first dose of the drug but on one occasion it did not occur until the 4th day. Recovery usually followed in 10-14 days. Interestingly, the increase in Ac-globulin concentration extended to values above the original levels, in one instance going as high as 150%, before returning to the normal range. This rise generally coincided with the period of marked prothrombin restoration. The dosage of 4 mg per kg of body weight approximates the original dose employed clinically in dicumarol therapy, though a total of 4 such doses is seldom utilized. In order to achieve maximal effects, 2 doses of 14 mg per kg were given to 5 of the dogs.

The prothrombin response to dicumarol in all dogs studied followed a pattern similar to that shown in Fig. 1, with the maximal reduction varying from 6% to less than 1% of normal. Both dosage schedules resulted in marked reductions of prothrombin. The smaller doses given over a greater period of time usually produced a slightly longer de-

[6] Seegers, W. H., and Ware, A. G., Josiah Macy, Jr., Conference on Blood Clotting and Allied Problems, N. Y., 1948.

[7] Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, **66**, 801.

pression of Ac-globulin.

2. *Human Patients.* Preliminary investigations were carried out on 8 patients to whom dicumarol was administered after the occurrence of intravascular thromboses. Prothrombin values were reduced under this therapeutic regime and maintained at the desired low levels. Whenever possible, samples for analysis were obtained from the patients before dicumarol therapy was instituted. The prothrombin and Ac-globulin levels were followed as systematically as possible in each case, usually at 2- or 3-day intervals.

These studies indicate that in the human being as well as in the experimental animal the Ac-globulin level is lowered following the first doses of dicumarol. The concentration may be reduced to half of that normally found in human beings. Some individual variation was observed. As the prothrombin concentration was brought to the desired level of about 20% and the dicumarol doses reduced to maintain it so, the Ac-globulin titer returned to normal values. This restoration was found to be complete after about 3 weeks of therapy. Even when followed into the fourth week of dicumarol administration none of these patients showed a concentration of Ac-globulin above normal. The prothrombin and Ac-globulin levels of a patient, recorded in Table I, are representative of our general experience.

In addition to those patients followed from a time prior to or early in therapy, plasma samples were also analyzed from 12 out-patients who had received dicumarol for periods ranging from 1 to 14 months. Prothrombin levels had been reduced as rapidly as was safely possible and for the most part were maintained within a range of between 15 and 30% of normal. These patients who had received dicumarol for long periods of time showed no appreciable variation in Ac-globulin from normal levels.

TABLE I.
Plasma Ac-globulin and Prothrombin Levels in a Human Patient Receiving Continuous Dicumarol Therapy.

| Days following initial administration of drug | Prothrombin % of normal | Ac-globulin % of normal |
|---|---|---|
| 1 | 77 | — |
| 2 | 34 | 49 |
| 3 | 56 | 52 |
| 5 | 35 | 49 |
| 7 | 24 | 68 |
| 9 | 20 | 59 |
| 12 | 21 | 64 |
| 14 | 26 | 67 |
| 16 | 26 | 89 |

*Discussion.* These observations in general agree with those of Owen and Bollman[8] who report on the effect of dicumarol in dogs. However, those authors do suggest a disappearance of the accelerator in the early stages of dicumarolization whereas no such marked alteration was found by us. The difference between the marked fall in accelerator indicated by their work and the relatively small decreases in Ac-globulin reported in this present study, even though higher dicumarol doses were utilized, may possibly be attributed to the methods of analysis used. The test developed in this laboratory is believed to be more sensitive to Ac-globulin and less sensitive to other factors in the plasma than is the method utilized by Owen and Bollman. The period of increased convertibility noted in their work in the recovery stages agrees with our observations which indicate that Ac-globulin concentrations in the canine species may reach 150% of the normal during that time.

Study of the Ac-globulin level in dogs is beset with certain problems which unless controlled may lead to erroneous results. The blood of a normal dog when removed from the vein clots rapidly. The Lee-White time of dog blood is 3-5 minutes compared to 6-9 minutes for normal human blood. If incipient initiation of the clotting process occurs in the sample to be tested the small amount of thrombin formed will partially convert the plasma Ac-globulin to the active form, serum Ac-globulin.[9] Experience has shown that this effect is easier to avoid when citrate is used as anticoagulant. The presence of a small amount of serum Ac-globulin will indicate an apparent but false increase in the plasma Ac-

[8] Owen, C. A., and Bollman, J. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 231.

[9] Ware, A. G., and Seegers, W. H., *Am. J. Physiol.*, 1948, **152**, 567.

globulin present.[9] On the other hand, if a considerable amount of thrombin is released prior to the effect of the anticoagulant it may actually destroy part of the Ac-globulin. To guard against these difficulties the procedure described in this paper for drawing blood was followed carefully. Sodium citrate is also preferable to oxalate as an anticoagulant because Ac-globulin is more stable in stored citrated dog plasma than in oxalated plasma.

It is certainly possible, or even probable, that the effect of dicumarol on the coagulation mechanism is not restricted to prothrombin and Ac-globulin alone. Conflicting reports[10,11] have been presented as to the susceptibility of fibrinogen to dicumarol and a variation in serum antithrombin titer has also been proposed.[12] From the observations reported here it is seen that dicumarol has, in addition to its paramount function of lowering the prothrombin concentration, a secondary effect upon the coagulation mechanism through its reduction of Ac-globulin early in the period of dicumarol therapy. The definite drop in Ac-globulin which occurs following initiation of therapy should contribute at that time to the prevention of clot formation.

*Summary.* In human patients receiving dicumarol the plasma Ac-globulin level may be depressed by 20-50% following initiation of therapy. Individual variation was noteworthy. A gradual return to normal concentrations of Ac-globulin occurs within 3 weeks as therapy is continued and the prothrombin is maintained at a low titer. No appreciable difference from normal was found in the Ac-globulin values of patients who had been on dicumarol therapy for 1 to 14 months.

Dogs receiving larger dicumarol doses than were administered to human patients showed a similar Ac-globulin response and a more marked reduction in prothrombin. A period of slightly lowered Ac-globulin activity in the dog is followed by a temporary rise to levels above normal.

[10] Irish, U. D., and Jaques, L. B., *Am. J. Physiol.*, 1945, **143**, 101.

[11] Peters, H. R., Doenges, J. P., and Brambel, C. E., *Southern Med. J.*, 1948, **41**, 526.

[12] Hurn, M., Barker, N. W., and Mann, F. D., *Am. J. Clin. Path.*, 1947, **17**, 712.

16766

## Indoleacetic Acid Studies in Man.*

HERBERT P. SARETT.

*From the Nutrition Research Laboratory, Department of Medicine, Tulane University School of Medicine, New Orleans.*

Corn products have usually been present in diets which have been associated with the etiology of pellagra in man,[1] blacktongue in dogs,[2] and nicotinic acid deficiency in rats.[3] It has been shown that the role of corn in the development of these deficiency syndromes is not due only to the low nicotinic acid content of the corn.[2,4,5] Other factors may be

* A preliminary report was presented at the meeting of the American Society of Biological Chemists, March, 1947. (*Fed. Proc.*, 1947, **6**, 288).

This work was aided by grants from the Nutrition Foundation, Inc., the Williams Waterman Fund of the Research Corporation, and the United States Public Health Service.

[1] Frazier, E. I., and Friedemann, T. E., *Quart. Bull. Northwestern Univ. Med. School*, 1946, **20**, 24.

[2] Handler, P., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 263.

[3] Krehl, W. A., Teply, L. J., and Elvehjem, C. A., *Science*, 1945, **101**, 283.

[4] Aykroyd, W. R., and Swaminathan, M., *Indian J. Med. Res.*, 1940, **27**, 667.

[5] Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A., *Science*, 1945, **101**, 489.

the low tryptophane content[5,6,7] and the possible presence of a "pellagragenic" agent[8,9] which could inhibit the utilization of nicotinic acid or the conversion of tryptophane to nicotinic acid.

The experiments on rats by Kodicek, Carpenter, and Harris[9] in 1946 suggested that the growth depressing or "pellagragenic" effect of corn may be due to its content of indole-3-acetic acid. In the present experiments the effect of indoleacetic acid supplements on nicotinic acid and tryptophane metabolism in man was evaluated by a study of the urinary excretion levels of nicotinic acid, tryptophane, and related metabolites, by subjects maintained on a controlled diet. While this work was in progress, other laboratories reported that indoleacetic acid had no effect on the growth of rats [10–12] or on the excretion of N′-methylnicotinamide (N′-Me) by rats.[12] Kodicek, Carpenter, and Harris[13] have also been unable to duplicate their earlier results with indoleacetic acid.

*Experimental.* The subjects were ward patients who were found to be free of complicating organic disease as determined by clinical and laboratory examinations. They were maintained in a separate metabolism ward and were permitted to be ambulatory. The wheat diet, which was used to maintain these subjects has been employed in other nicotinic acid and tryptophane studies.[7,14] This diet, which was planned to provide 2400 calories, contained 248 g of unenriched wheat products and supplied 43 g of protein, 430 mg of tryptophane, and 6 mg of nicotinic acid per day. Only 10% of the total protein was of animal origin. Variations in food intake, which were necessary in order to adjust the caloric intake, were recorded. After suitable control periods on these diets, indole-3-acetic acid (Eastman) was added as a supplement with dinner and supper.

Twenty-four-hour urines were collected in amber bottles containing 15 ml of glacial acetic acid, stored in the refrigerator and pooled in 48-hour periods for analysis of creatinine, nitrogen, thiamine, riboflavin, nicotinic acid, N′-Me, and tryptophane by methods previously described.[14] Since it has been observed that in rats and humans who had received tryptophane supplements, the apparent nicotinic acid content of the urine was markedly increased by autoclaving with strong acid,[14,15] the urines in two of the present experiments were autoclaved in the presence of 1 *N* $H_2SO_4$ for 30 minutes prior to assay of nicotinic acid. Another metabolite, which appeared in the urine of subjects who had received extra tryptophane, has tryptophane-like activity for *L. arabinosus* but is extractable from urine (at pH 4) by ether.[14] Ether extraction was tried in a few of the present experiments in an effort to detect any increase in this metabolite after indoleacetic acid administration.

Indoleacetic acid was estimated only in those urines obtained after administration of this substance, using the method suggested by Tang and Bonner.[16] In this test tryptophane gave 0.7% of the color obtained with indoleacetic acid while indole produced 14% and xanthurenic acid 1.3% of the indoleacetic acid color. About 10 to 15 mg of indoleacetic acid per day was found by this method in urines collected from the subjects during the basal period. Since many indole derivatives which appear in urine may interfere with this test, these basal values were not considered to be reliable.

6 Singal, S. A., Sydenstricker, V. P., and Littlejohn, J., *Fed. Proc.*, 1947, **6**, 422.

7 Sarett, H. P., and Goldsmith, G. A., *J. Biol. Chem.*, 1947, **167**, 293.

8 Woolley, D. W., *J. Biol. Chem.*, 1946, **163**, 773.

9 Kodicek, E., Carpenter, K. J., and Harris, L. J., *Lancet*, 1946, **2**, 491.

10 Krehl, W. A., Henderson, L. M., de la Huerga, J., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, **166**, 531.

11 Krehl, W. A., Carvalho, A., and Cowgill, G. R., *Fed. Proc.*, 1947, **6**, 413.

12 Rosen, F., and Perlzweig, W. A., *Arch. Biochem.*, 1947, **15**, 111.

13 Kodicek, E., Carpenter, K. J., and Harris, L. J., *Lancet*, 1947, **2**, 616.

14 Sarett, H. P., and Goldsmith, G. A., *J. Biol. Chem.*, in press.

15 Singal, S. A., Briggs, A. P., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, 1946, **166**, 573.

16 Tang, Y. W., and Bonner, J., *Arch. Biochem.*, 1947, **13**, 11.

TABLE I.

Effect of Indoleacetic Acid Administration on Daily Excretion of Tryptophane, Nicotinic Acid, and Related Compounds by Subject I.

| Diet per day | Days | Creatinine, g | Nitrogen, g | Thiamine, γ | Riboflavin, γ | Nicotinic acid, mg | N'-Me, mg | Tryptophane, mg |
|---|---|---|---|---|---|---|---|---|
| Wheat (2520 calories, 44 g protein) | 1-2 | 1.1 | 5.9 | 71 | 470 | 0.5 | 1.6 | 11 |
| | 3-4 | 1.1 | 5.6 | 44 | 235 | 0.4 | 1.2 | 11 |
| | 5-6 | 1.2 | 5.4 | 50 | 150 | 0.5 | 1.4 | 13 |
| | 7-8 | 1.2 | 5.0 | 30 | 115 | 0.4 | 1.3 | 12 |
| Wheat + 25 mg indoleacetic acid | 9-10 | 1.3 | 5.8 | 33 | 107 | 0.5 | 1.6 | 13 |
| | 11-12 | 1.3 | 5.9 | 29 | 104 | 0.5 | 1.6 | 13 |
| | 13-14 | 1.2 | 5.7 | 20 | 53 | 0.5 | 1.2 | 14 |
| Wheat + 50 mg indoleacetic acid | 15-16 | 1.2 | 5.7 | 22 | 52 | 0.5 | 1.1 | 13 |
| | 17-18 | 1.2 | 5.8 | 21 | 52 | 0.5 | 1.5 | 14 |
| | 19-20 | 1.1 | 5.2 | 20 | 56 | 0.4 | 1.3 | 12 |
| Wheat + 100 mg indoleacetic acid | 21-22 | 1.2 | 5.3 | 19 | 65 | 0.5 | 1.2 | 15 |
| | 23-24 | 1.2 | 5.6 | 25 | — | 0.6 | 1.3 | 16 |
| | 25-26 | — | — | — | — | — | — | — |
| Wheat | 27-28 | 1.0 | 4.9 | 16 | 61 | 0.6 | 1.1 | 11 |
| | 29-30 | 1.1 | 5.5 | 21 | 80 | 0.6 | 1.3 | 15 |
| | 31-32 | 1.0 | 5.7 | 21 | 66 | 0.5 | 1.8 | 14 |
| | 33-34 | 1.1 | 5.8 | 27 | 74 | 0.5 | 1.8 | 15 |

TABLE II.

Effect of 200 mg of Indoleacetic Acid on Daily Excretion of Tryptophane, Nicotinic Acid, and Related Compounds by Subject I.

| Diet per day | Days | Nicotinic acid | | | Tryptophane | | Indoleacetic acid, mg |
|---|---|---|---|---|---|---|---|
| | | As is, mg | Acid hydrol., mg | N′-Me, mg | As is, mg | After ether extr., mg | |
| Wheat (2530 calories, 44 g protein) | 1-2 | 0.7 | 1.1 | 2.3 | 16 | | |
| | 3-4 | 0.5 | 0.8 | 1.3 | 12 | | |
| | 5-6 | 0.5 | 1.1 | 1.5 | 13 | | |
| | 7-8 | 0.5 | 1.0 | 1.2 | 13 | 12 | |
| Wheat + 200 mg indoleacetic acid | 9-10 | 0.7 | 1.4 | 1.3 | 18 | 17 | 130 |
| | 11-12 | 0.6 | 1.2 | 1.4 | 18 | 17 | 138 |
| | 13-14 | 0.5 | 1.2 | 1.4 | 17 | | — |
| | 15-16 | 0.6 | 1.7 | 1.9 | 16 | | 83 |
| Wheat | 17-18 | 0.7 | 1.8 | 1.9 | 13 | | |
| | 19-20 | 0.5 | 0.9 | 1.4 | 12 | | |
| | 21-22 | 0.6 | 1.2 | 1.2 | 15 | | |

TABLE III.

Effect of 200 mg of Indoleacetic Acid on Daily Excretion of Tryptophane, Nicotinic Acid, and Related Compounds by Subject II.

| Diet per day | Days | Nicotinic acid | | | Tryptophane | | Indoleacetic acid, mg |
|---|---|---|---|---|---|---|---|
| | | As is, mg | Acid hydrol., mg | N′-Me, mg | As is, mg | After ether extr., mg | |
| Wheat (2370 calories, 41 g protein) | 1-2 | 0.6 | 1.2 | 2.7 | 6 | | |
| | 3-4 | 0.4 | 0.9 | 1.7 | 4 | | |
| | 5-6 | 0.6 | 1.3 | 2.4 | 5 | | |
| | 7-8 | 0.5 | 1.2 | 2.2 | 5 | 4 | |
| Wheat + 200 mg indoleacetic acid | 9-10 | 0.6 | 1.3 | 2.1 | 6 | 5 | 70 |
| | 11-12 | 0.5 | 1.2 | 2.1 | 6 | 5 | 97 |
| | 13-14 | 0.6 | 1.3 | 1.8 | 6 | | 73 |
| | 15-16 | — | — | — | — | | — |
| Wheat | 17-18 | 0.5 | 1.5 | 1.3 | 4 | | |
| | 19-20 | 0.5 | 1.1 | 1.6 | 4 | | |
| | 21-22 | 0.5 | 1.2 | 1.5 | 5 | | |

*Results.* The values obtained in the analyses of the urine in these experiments are presented in Tables I, II and III. The subject for the experiments shown in Table I, and III was a 30-yr.-old male (W.H.) and in Table II a 47-yr.-old female (J.H.) The subjects were maintained on the basal wheat diet for 8 days before the supplements of 25 to 200 mg of indoleacetic acid were given. Since corn is reported to contain 20 to 100 mg of indoleacetic acid per kg[17,18] 25 mg of indoleacetic acid represents that found in 0.25 to 1.2 kg of corn while 200 mg of indoleacetic acid is equivalent to that obtained from 2 to 10 kg of corn products.

The creatinine and nitrogen excretion remained fairly constant in each experiment and are an indication of the completeness of urine collection and the constancy of the diet. The levels of thiamine and riboflavin excreted decreased rapidly, as has been found with other subjects on this diet,[14] and were not affected by the indoleacetic acid administration. For the sake of brevity, the above findings are shown only for the experiment in Table I.

The urinary nicotinic acid values obtained by microbiological analysis of the diluted urine were not significantly changed throughout the course of the experiments. The nicotinic acid values which were obtained after autoclaving the urine with acid (Tables II and III) also remained relatively constant. The slight increase in excretion of this metabolite at the end of the period of indoleacetic acid administration (Table II) is of questionable significance. Tryptophane is the only known compound which, when added to the diet, leads to an increase in excretion of this acid-hydrolyzable nicotinic acid-like compound.[14,15]

The excretion of N′-Me is at present the main index of nicotinic acid metabolism. In the present experiments there were no significant changes in excretion of N′-Me after administration of indoleacetic acid. In previous studies it has been shown that replacement of 190 g of the wheat of this diet by corn products resulted in a marked decrease in excretion of N′-Me.[7,14] This may have been due to the presence of an inhibitor in the corn,[8] or to the lower tryptophane content of the corn as compared with the wheat, or to a combination of these factors.

Tryptophane excretion increased slightly in all 3 experiments following the administration of indoleacetic acid and decreased to approximately the basal level after the supplementation was discontinued. The presence of indoleacetic acid in the urine could not account for the increased tryptophane values. Relatively high levels of indoleacetic acid (0.1 mg/ml) in the microbiological assay have been shown to have a slight stimulatory effect upon the utilization of tryptophane by *L. arabinosus*.[19] However, urines which were diluted for analysis of tryptophane, supplied less than 50 γ of indoleacetic acid for each 10 ml assay tube. Experiments in this laboratory show that 20 of 500 γ of indoleacetic acid per 10 ml assay tube has no activity for *L. arabinosus* in a test which can detect less than 0.2 γ of tryptophane and has no significant effect on tryptophane utilization. However, the metabolism of indoleacetic acid, either in the intestine or in the body, may lead to the formation of other compounds which can replace tryptophane or stimulate the utilization of tryptophane by *L. arabinosus*. Only 70 to 138 mg of indoleacetic acid were found in the urine after oral administration of 200 mg of indoleacetic acid (Tables II and III).

Extraction of some of the urines with ether showed (Tables II and III) that the increase in tryptophane excretion after indoleacetic acid supplementation was not due to the ether extractable tryptophane-like substance which is found in the urine after tryptophane administration.[14]

*Discussion.* In the present experiments the addition of up to 200 mg of indoleacetic acid per day to a low protein diet appeared to have no significant effect on nicotinic acid utilization or on the conversion of trypto-

[17] Haagen-Smit, A. J., Leech, W. D., and Bergren, W. R., *Am. J. Botany*, 1942, **29**, 500.

[18] Berger, J., and Avery, G. S., *Am. J. Botany*, 1944, **31**, 199.

[19] Handler, P., and Kamin, H., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 251.

phane to nicotinic acid as evidenced by the excretion of nicotinic acid and N′-Me by man. Rosen and Perlzweig[12] showed that indoleacetic acid had no effect on the excretion of N′-Me by rats receiving a basal diet alone or with tryptophane supplements. Others have also been unable to obtain depression of rat growth by inclusion of indoleacetic acid in the diet, although the addition of corn or protein low in tryptophaone has retarded growth.[10–13] The replacement of a portion of the wheat diet by corn products has been shown to decrease the excretion of N′-Me by man.[7,14] This effect may be due to the lower tryptophane content of the corn as compared with the wheat or to the presence of an unidentified inhibitory agent in the corn.[8]

The increase in excretion of tryptophane compounds by man after the ingestion of indoleacetic acid may be due to the conversion of indoleacetic acid to a substance which has tryptophane-like activity for *L. arabinosus*. It is also possible that indoleacetic acid may have some effect on tryptophane metabolism in man which is separate from the tryptophane-nicotinic acid relationship. In rats, indoleacetic acid cannot replace tryptophane as a growth stimulant, when added to a diet deficient in tryptophane and nicotinic acid.[10–13]

*Summary.* The addition of 25 to 200 mg of indoleacetic acid per day to a diet low in protein and nicotinic acid appears to have no significant effect upon the utilization of tryptophane and nicotinic acid by man as evidenced by urinary excretion studies.

The author wishes to thank the following people for their cooperation in these studies: Drs. Grace A. Goldsmith and Roy E. Butler for clinical assistance, Janice Gibbens and Winifred Bouvet for planning and supervising the diets, and Antoinette Dingraudo, Carol Haas and Janice Loeb for their technical assistance.

16767 P

## Changes in Circulating Eosinophils in Man Following Epinephrine, Insulin, and Surgical Operations.*

JOHN H. LARAGH AND THOMAS P. ALMY. (Introduced by Ephraim Shorr.)
*From the Department of Medicine, New York Hospital, and the Cornell University Medical College, New York City.*

Forsham and his associates[1] have reported that pituitary adrenocorticotropic hormone (ACTH) produces a fall in circulating eosinophils which is dependent upon normal adrenal cortical function. Long and Fry[2] had previously demonstrated that epinephrine stimulates the adrenal cortex of the rat, and that this effect is mediated by the anterior pituitary. For these reasons we have studied the effects upon the circulating eosinophils of epinephrine, and later of insulin and of surgical operations. Studies have been made upon healthy persons, patients with adrenal and pituitary insufficiency, and patients with "diseases of adaptation"[3] such as essential hypertension and nonspecific colitis.

While this study was in progress, eosinopenia after intravenous epinephrine and insulin was reported by Godlowski,[4] and Thorn and his associates[5] reported that intravenous epinephrine reduced the eosinophil count in

* Aided by a generous gift from Mr. John L. Given.

1 Forsham, P. H., Thorn, G. W., Prunty, F. T. G., and Hills, A. G., *J. Clin. Endocrinol.*, 1948, **8**, 15.

2 Long, C. N. H., and Fry, E. G., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 67.

3 Selye, H., *J. Clin. Endocrinol.*, 1946, **6**, 117.

4 Godlowski, Z. Z., *Brit. Med. J.*, 1948, **1**, 46.

5 Recant, L., Forsham, P. H., and Thorn, G. W. Read at the thirtieth meeting of the Association for the Study of Internal Secretions, June 18, 1948.

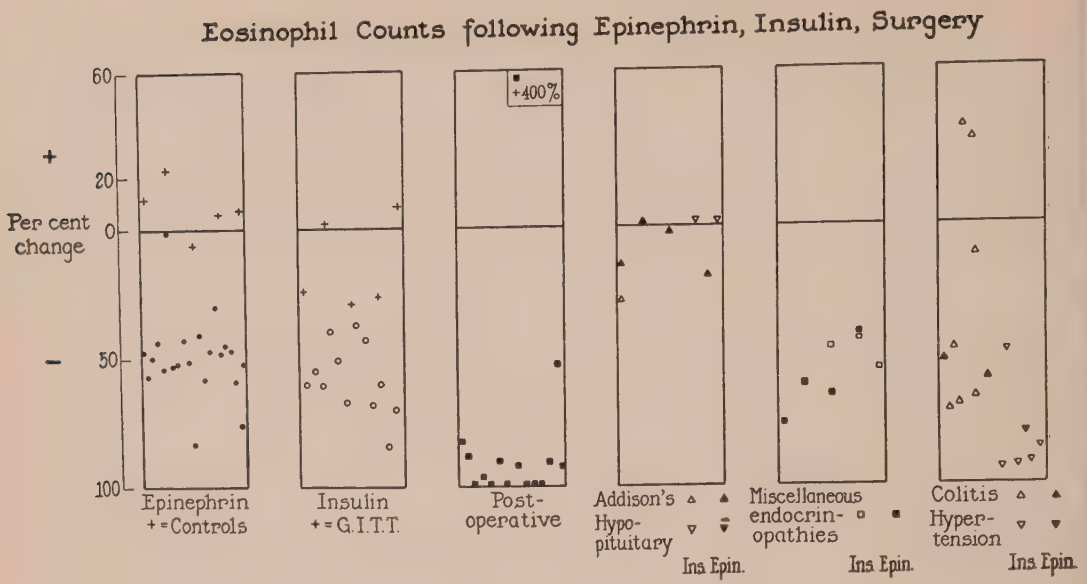



FIG. 1.

persons with normal adrenals. Gershberg and Long[6] demonstrated that insulin stimulated the adrenal cortex of animals with intact pituitaries.

*Method.* The eosinophils of heparinized venous blood were enumerated in the Levy double depth counting chamber, using a modification of the method of Dunger.[7]

*Results* (see Figure). The normal range of eosinophil count was found to be 100-300 per cu mm.

*Epinephrine.* Epinephrine HCl, 0.5 cc of 1:1000 solution, was administered subcutaneously to 21 healthy subjects. In 20 of these subjects the eosinophil count dropped by an average of 56% in 2½ - 3 hours. Five subjects given 0.5 cc saline showed no significant drop in eosinophils.

*Insulin.* Regular insulin 0.1 unit/kg was given intravenously to 11 healthy subjects and to one patient with moderately advanced pulmonary tuberculosis who was on prolonged bed rest. In all these subjects the eosinophil count fell, the average change being minus 57.5%, four hours after injection. In all cases a blood sugar of 60 mg% or below was attained. Five subjects were given standard glucose-insulin tolerance tests as controls. Two of these patients showed no significant change; the other 3 had slight drops (22 to 28%) but in these 3 we were unable to obtain neutral sugar curves.

*Addison's Disease and Pituitary Insufficiency.* In 4 cases of Addison's Disease and 2 cases of pituitary insufficiency the typical fall in eosinophil count after epinephrine and insulin did not develop. The largest change observed was a 28% fall, associated with sustained hypoglycemia in a patient with Addison's Disease.

*Miscellaneous Endocrinopathies.* In this group are 2 acromegalics, 2 cases of pituitary tumor, 1 of Cushing's syndrome, and 1 of possible Cushing's. This group all responded normally with an average drop of 55.2% after epinephrine or insulin.

*Hypertension.* Six patients with essential hypertension have been studied. Four showed an exaggerated response to insulin with an average drop in eosinophils of 90.4%. Blood sugar curves were similar to those observed in the healthy group. A fifth mild hypertensive dropped 79% after epinephrine and the sixth patient showed a 48% drop after insulin.

*Colitis.* Of 3 patients with severe ulcera-

6 Gershberg, H., and Long, C. N. H., *ibid.*

7 Dunger, A., *Munchen. Med. Wchnschr.*, 1910, **57**, 1942.

tive colitis given insulin, 2 showed a rise in eosinophils (32%, 37%) and a third only a 12% drop. Five patients with milder ulcerative colitis showed a normal response.

*Surgical Operations.* Fifteen patients have been followed through the stress of major surgery. Following operation, 14 of these patients showed profound drops in their eosinophil counts, 6 falling to zero. In general the degree of fall varied directly with the magnitude of the operation. The peak of response occurred most commonly 3-8 hours after the beginning of the operation. The fifteenth patient, one with severe ulcerative colitis, showed a marked and progressive eosinophilia following an ileostomy.

*Discussion.* Epinephrine and insulin appear to exert similar effects on the circulating eosinophils of man, and our results suggest that this effect is mediated through the pituitary and adrenal cortex. The failure of response in a small number of cases of Addison's Disease and pituitary insufficiency leads us to anticipate that the eosinophil changes following epinephrine and insulin may be useful in the diagnosis of these conditions.

Browne and his associates[8] have measured the changes in excretion of glycogenic corticoids and other indices of adrenal function following major surgery, and have interpreted these changes as part of the process of adaptation to non-specific stress. The occurrence of eosinopenia following operations may be construed as further evidence of the role of the adrenal cortex in the "alarm reaction" in man.

In view of the postulated role of the adrenal in the genesis of "essential" hypertension, the exaggerated eosinophil response in patients with this disorder is being studied further. The absence of an eosinophil response in three patients with severe chronic ulcerative colitis suggests that adrenal insufficiency may occur in such patients. This possibility is being investigated.

*Summary.* Significant eosinopenia has been observed following injection of epinephrine and of insulin. This change was exaggerated in patients with essential hypertension, and diminished or absent in patients with adrenal or pituitary insufficiency. Marked eosinopenia has been found after surgical operations.

The authors are indebted to Dr. Peter Forsham for technical advice.

8 Browne, J. S. L., Conference on Bone and Wound Healing, Josiah Macy, Jr., Foundation, December 11-12, 1942, pp. 45-47.

16768

## Electrophoretic Patterns After Dicumarol Medication.

SHEPARD SHAPIRO AND DAN H. MOORE.

*From the Goldwater Memorial Hospital, the Department of Medicine, N. Y. University, College of Medicine, and the Electrophoresis Laboratory, College of Physicians and Surgeons, New York City.*

Dicumarol induces prothrombinopenia by inhibiting the production of prothrombin, presumably in the liver. At dosage levels used for therapeutic purposes, its action is reversible and can be counteracted by vitamin K.[1-3] In order to determine whether or not protein components other than prothrombin were measurably altered by the drug, comparisons by means of electrophoresis of human plasma before and after Dicumarol medication were made.

1 Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 1.

2 Shapiro, S., Redish, M. H., and Campbell, H. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, **12.**

3 Cromer, H. E., Jr., and Barker, N. W., *Proc. Staff Meet. Mayo Clin.*, May, 1944, **19**, 217.

TABLE I.
Electrophoretic Data and Prothrombin Times on Plasma from Patients after Dicumarol.

| Specimen | % concentration of | | | | | A/G | Prothrombin time |
|---|---|---|---|---|---|---|---|
| | Albumin | Globulins | | | | | Whole plasma, sec. |
| | | α | β | Φ | γ | | |
| Ma I | 57.8 | 8.5 | 10.0 | 8.2 | 15.4 | 1.37 | 17.5 |
| Ma II | 59.3 | 8.2 | 11.9 | 6.7 | 13.9 | 1.45 | 39.5 |
| Mo I | 66.8 | 7.2 | 14.2 | 3.5 | 8.5 | 2.00 | 16.0 |
| Mo II | 66.2 | 7.6 | 13.9 | 3.8 | 8.3 | 1.97 | 40.6 |
| Ox I | 65.1 | 5.7 | 11.4 | 4.5 | 13.3 | 1.86 | 15.0 |
| Ox II | 64.4 | 4.5 | 12.2 | 4.5 | 14.4 | 1.81 | 38 |
| Ow I | 45.9 | 11.6 | 18.0 | 9.1 | 15.2 | 0.85 | 15.2 |
| Ow II | 42.8 | 12.0 | 17.3 | 9.1 | 18.8 | 0.76 | 23.0 |
| Me I | 49.7 | 8.6 | 21.0 | 6.9 | 13.8 | 0.99 | 15.2 |
| Me II | 49.7 | 6.6 | 21.8 | 9.1 | 12.8 | 0.98 | 37.6 |
| Kr I | 54.1 | 9.9 | 13.4 | 5.3 | 17.4 | 1.18 | 13.8 |
| Kr II | 56.2 | 9.2 | 12.5 | 4.5 | 17.6 | 1.28 | 23.5 |

Mo and Ox = Normal subjects.
I = Before medication (dicumarol).
II = 48 hr later.

*Material.* The plasmas of 6 adult persons, 3 of each sex, were studied. Four were patients being treated for intravascular thrombosis, and 2 were normal subjects. The doses of Dicumarol used in each case varied between 500 and 700 mg given in 2 doses about 12 hours apart, the first dose being usually about 100 mg greater than the second. Prothrombin estimations were made immediately prior to the administration of the initial dose and again about 48 hours later. Samples of blood for electrophoresis were obtained at the same time.

Estimations of prothrombin time were made by a method previously described.[4]

Since hemoglobin migrates electrophoretically with γ-globulin, hemolysis was carefully avoided. Plasmas from oxalated blood samples were diluted with 2 volumes of 0.02M sodium phosphate buffer of pH 7.4 which was 0.15M with respect to NaCl, ionic strength 0.2, and dialyzed in the cold against the same buffer solution for one day. Analyses were made in the Tiselius apparatus in the usual manner.

*Results.* Planimeter measurements and the calculated percentages of the several plasma components are recorded in Table I It is apparent from this table, that no consistent changes occurred in the electrophoretic patterns although there was marked Dicumarol-induced prothrombinopenia. This was true for samples of plasma taken both for 48 and 72 hours after Dicumarol administration. The quantity of prothrombin in plasma is too small to be demonstrated by electrophoresis, therefore, no evidence of its depression was expected in the patterns. Only one patient, Kr, exhibited a significant change in A/G ratio. Before medication, the value of this ratio was 1.18. It rose to 1.28 after 48 hours and dropped to 0.95 when estimated 24 hours later. The fall was a reflection of a change in all components. This patient had generalized arteriosclerosis, circulatory failure and peripheral venous thrombosis. It is possible that the disease processes were responsible for this change. We hesitate to ascribe it to the influence of the drug.

*Summary.* Dicumarol at therapeutic dosage levels failed to produce alterations in human plasma of sufficient magnitude to be detected by electrophoretic analysis.

[4] Shapiro, S., Sherwin, B., Redish, M., and Campbell, H. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 85.

16769

# Comparison of New Jersey and Palestine Strains of Bovine Leptospira.

H. Bernkopf* and Ralph B. Little. (Introduced by Carl TenBroeck.)

*From the Department of Animal and Plant Pathology of the Rockefeller Institute for Medical Research, Princeton, N. J.*

In the previous work on leptospirosis in cattle in New Jersey[1,2] the similarity to the disease reported in Palestine[3-5] was obvious, but no culture of the Palestine strain was at hand for serological comparisons. The present paper reports serological studies carried out with 6 strains of leptospira isolated in an outbreak of the disease in a New Jersey dairy herd and a strain of bovine leptospira from Palestine.

*Methods.* Chang's[6] semisolid and fluid media (in which rabbit serum had been substituted for horse serum and the hemoglobin omitted) were used for storing cultures and Schueffner's[7] medium was used for the serological tests. Actively growing cultures incubated 5 to 7 days at 30°C were used as antigen either as living or formalinized preparations. Equal amounts of serum dilutions and leptospira suspensions were mixed in small test tubes. For agglutination tests formalinized cultures (0.3% formalin) were incubated for 2 hours at 37° C and kept at room temperature, while with living antigen the incubation period was the same but the tubes were stored in the refrigerator. Readings were made the following morning by examining uncovered drops by dark-field illumination with objective 10 and ocular 10. The results with living cultures were recorded as +, all organisms lysed; ± many organisms lysed; and —, no lysis. With formalinized material the readings were recorded as +, nearly all leptospiras agglutinated in large clumps; ±, many leptospiras agglutinated in small clumps; and —, no agglutination.

*Serological Examination.* In the serological studies it was found that both living and formalinized cultures gave identical titers as a rule, yet is was useful to check the results of one method with the other. Small antibody concentrations may not be detected if living cultures are employed, as minor degrees of lysis are more difficult to read than slight agglutination reactions obtained with formalinized material. The disadvantage, however, of using live cultures is offset by the avoidance of spontaneous clumping which not infrequently occurs in formalinized cultures.

Three leptospiral strains (N.J.) were used to immunize rabbits. The rabbits received 3 intravenous injections of living cultures at weekly intervals and were bled one week after the last injection. Each New Jersey immune serum agglutinated the 3 New Jersey strains to the same titer. It was therefore assumed that the strains were identical and this was confirmed in absorption experiments. Subsequently 2 rabbits were immunized, one with a New Jersey culture, the other with the Palestine strain. Each animal received 4 injections of living culture intravenously and a week after the last injection they were bled from the heart. The results for both agglutination and

* Visiting investigator from the Department of Hygiene and Bacteriology, The Hebrew University, Jerusalem.

1 Baker, J. A., and Little, R. B. Presented in abstract form before the Twenty-eighth Conference of Research Workers in Animal Diseases in North America, Chicago, Ill., Dec. 2, 1947. Also in Bovine Mastitis. A Symposium (R. B. Little and W. N. Plastridge, editors), New York, McGraw-Hill Book Co., Inc., 1946.

2 Baker, J. A., and Little, R. B., *J. Exp. Med.*, 1948, **88**, 295.

3 Bernkopf, H., Olitzki, L., and Stuczynski, L. A., *J. Infect. Dis.*, 1947, **80**, 53.

4 Ungar, H., and Bernkopf, H., *Arch. Path.*, 1947, **44**, 59.

5 Bernkopf, H., Stuczynski, L. A., Gottlieb, T., and Halevy-Katz, C., *J. Infect. Dis.*, 1948, in press.

6 Chang, Shih Lu, *J. Infect. Dis.*, 1947, **81**, 28.

7 Kelser, R. A., and Schoening, H. W., Manual of Veterinary Bacteriology, 4th Edition, Williams and Wilkins Co., Baltimore, Md., 1943, p. 411.

TABLE I.

Agglutination and Lysis Tests with Immune Sera Prepared in Rabbits Against the New Jersey and the Palestine Strains of Leptospira.

| Strain | Antigen used | Antiserum to New Jersey strain | | | | | Antiserum to Palestine strain | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1:20 | 1:200 | 1:2,000 | 1:20,000 | 1:200,000 | 1:20 | 1:200 | 1:2,000 | 1:20,000 | 1:200,000 |
| New Jersey | Formalinized culture | + | + | + | ± | — | — | — | — | — | — |
| | Living culture | + | + | + | ± | — | — | — | — | — | — |
| Palestine | Formalinized culture | ± | ± | — | — | — | + | + | + | ± | — |
| | Living culture | ± | — | — | — | — | + | + | + | ± | — |
| *L. icterohaemorrhagiae* | Formalinized culture | — | — | — | — | — | ± | ± | — | — | — |
| | Living culture | — | — | — | — | — | — | — | — | — | — |
| *L. canicola* | Formalinized culture | — | ± | — | — | — | — | ± | — | — | — |
| | Living culture | — | — | — | — | — | — | — | — | — | — |

TABLE II.

Examination of Sera of Two New Jersey Cows* for Antibodies Against the New Jersey and Palestine Strains of Leptospira.

| Cow No. | Date of onset of illness | Date of bleeding | Agglutination of formalinized culture | | | | | | | | Lysis of living culture | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | New Jersey strain Dilution of sera | | | | Palestine strain Dilution of sera | | | | New Jersey strain Dilution of sera | | | | Palestine strain Dilution of sera | | | |
| | | | 1:20 | 1:200 | 1:2000 | 1:20,000 | 1:20 | 1:200 | 1:2000 | 1:20,000 | 1:20 | 1:200 | 1:2000 | 1:20,000 | 1:20 | 1:200 | 1:2000 | 1:20,000 |
| 1 | 12/2/46 | 12/2/46 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | | 1/9/47 | ± | + | + | — | — | — | — | — | ± | + | + | — | — | — | — | — |
| 2 | 4/6/48 | 4/6/48 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | | 4/26/48 | + | + | + | — | + | — | — | — | + | + | + | — | — | — | — | — |

* Bled at the onset of the disease and after recovery.

TABLE III.
Inoculation of the New Jersey Strain into Guinea Pigs Previously Inoculated with the Palestine Strain.

| Guinea pig No. | First inoculation | Results of second inoculation with New Jersey strain |
|---|---|---|
| 1 | Palestine strain | Fever after 3 days |
| 2 | " | " " 6 " |
| 3 | " | " " 7 " |
| 4 | " | " " 7 " |
| 5 | " | No fever |
| 6 | " | " " |
| 7 | " | " " |
| 8 | Not inoculated | Fever after 2 days |
| 9 | " " | " " 3 " |
| 10 | " " | " " 3 " |
| 11 | " " | " " 3 " |

lysis tests are given in Table I. Strains of *Leptospira canicola* and *Leptospira icterohaemorrhagiae* kindly supplied by Dr. R. E. Kelser were also included in this test.

It can be seen in Table I that no marked cross reactions occurred with the New Jersey and Palestine strains. Antiserum to the Palestine strain did not cross react with the New Jersey strain, while the antiserum to the New Jersey strain showed a weak cross reaction with the Palestine strain in a dilution of 1:20 (lysis) and 1:200 (agglutination). It is also clearly shown in Table I that serologically both bovine strains are different from *L. icterohaemorrhagiae* and *L. canicola.*

*Examination of sera from naturally infected cows.* Sera from cows of the New Jersey herd which had recovered from an attack of the disease reacted strongly with the New Jersey strain[1,2] but showed little if any reaction against the Palestine culture.[3] The reaction of the sera of 2 cows taken at the onset of the disease and 20 and 38 days later, respectively, is given in Table II. The specificity of the reaction with the New Jersey strain is clearly shown, for neither serum from the recovered animals agglutinated the Palestine culture.

The sera of 10 cows which had shown typical symptoms of the disease in May 1946 (bled 2 to 3 weeks after the onset of the illness) reacted with the New Jersey culture in dilutions up to 1:20,000. One serum agglutinated the Palestine strain in a dilution of 1:2,000. Of the bovine sera tested from both normal and recovered cases in the New Jersey herd, this was the only serum that agglutinated the Palestine culture in any dilution.

Forty-six sera from cows in a barn in which 2 typical cases of abnormal milk (bloody or thick off-colored milk) had occurred 2 months earlier were tested against both strains. Eight of the sera gave a positive reaction with the New Jersey strain in a dilution of 1:200, and 4 in a 1:2,000 dilution, thus suggesting that inapparent infections are not uncommon.

*Pathogenicity of the Strains for Guinea Pigs.* A cross infection experiment was set up by inoculating guinea pigs first with the Palestine strain and 3 weeks later with a New Jersey strain isolated from the milk of cow No. 1489. Each animal received intraperitoneally one ml of a leptospira culture in fluid medium. The results are given in Table III.

The guinea pigs showed no reaction following the injection of the Palestine strain, confirming earlier observations.[3] The New Jersey strain, however, produced fever in all control animals 2 to 3 days after the inoculation.[2] Only one of the 7 guinea pigs, which had received the Palestine strain before, developed fever 3 days after inoculation with the New Jersey strain. Three guinea pigs of the same group became febrile after an incubation period of 6 to 7 days (nearly twice as long as in the controls) while the remaining animals appeared normal during the entire period of observation. It is obvious therefore that, based on the development of fever, infection with the Palestine strain had produced complete immunity in 3 guinea pigs and partial immunity in 3 other animals out of a total of 7 tested.

A comparison of Tables I and III shows that the serological examination, because it is quantitative, gave a better differentiation of the strains. Schueffner and Mochtar[8] found in numerous experiments that guinea pigs that had recovered from infection with one strain of leptospira were protected against strains which serologically were unrelated.

8 Schueffner, W., and Mochtar, A., *Z. f. Bakt. Abt. 1, Orig.*, 1926-27, **101**, 405.

*Summary.* A study has been made of strains of leptospira recovered from an outbreak of leptospirosis among cattle in New Jersey and a strain isolated from an outbreak of the disease in Palestine. Sera from recovered cattle as well as sera from immunized rabbits were used in agglutination and lysis tests and it was found that all the New Jersey strains reacted alike while the Palestine strain belonged to another serological group.

16770

## Effect of Antihistamines on Loss of Adhesiveness of Corneal Epithelium After Injection of Histamine.

HEINZ HERRMANN. (Introduced by J. S. Nicholas.)

*From Osborn Zoological Laboratory, Yale University, New Haven, Conn.*

In a previous paper[1] we described the effect of histamine on the adhesiveness of the corneal epithelium of excised bovine corneas. In amounts from 0.5 to 1.0 $\mu$g per cornea (600-800 mg wet weight) histamine caused a marked decrease in epithelial adhesiveness. In contrast to other agents which decrease adhesiveness by disruption at the stroma epithelium boundary, histamine seems to effect the detachment of the upper layers of the epithelium from the basal one.

In view of the relatively high amounts of histamine required for this effect (about 10 - 100 times more than for relaxation of cats intestine) and of the gradual culmination of epithelial loosening it seemed desirable to compare the action on the corneal epithelium of histamine alone and of mixtures of histamine with certain antihistamines. The same technic was used as described previously.[2] Between 0.04 to 10 $\mu$g of the antihistamines were injected together with a constant amount of 2 $\mu$g of histamine in a total volume of 0.2 ml per cornea. At this concentration the antihistamines were found to have no effect on the adhesiveness of the corneal epithelium. On injection of 50 $\mu$g per cornea or more, toxicity with loosening of the epithelium was observed for all antihistamines tested so far. The indicated amount of histamine was sufficient to cause loosening in all samples. The corneas were incubated for 12 - 15 hours at 28 - 30°C. The adhesiveness was tested with a simple scraper which allows a semi-quantitative determination of the adhesiveness, the amount of removable epithelium being a function of the adhesiveness and of the weight on the scraping blade.[2] The procedure was simplified in the present series of experiments by keeping the pressure on the scraper constant (60 g) and recording the removal of more than 2/3 and of less than 1/3 of the epithelium of the corneal test strip. After injection of histamine alone there was always under these conditions a removal of more than 2/3 of the epithelium. If antihistamines were injected simultaneously the loss of adhesiveness was reduced to less than 1/3 depending upon the concentration of the respective compound.

In Table I our results are summarized designating protection with removal of less

1 Herrmann, H., *Bull. Johns Hopkins Hosp.*, 1948, **82**, 208.

2 Herrmann, H., and Hickman, F. H., *Bull. Johns Hopkins Hosp.*, 1948, **82**, 182.

TABLE I.
Antagonism to Loosening of Corneal Epithelium After Injection of Histamine in its Dependence Upon Concentration of Antihistamines.

| Amt of antihistamine per cornea in $\mu$g | Pyribenzamine | PTDA (01013) | Antistine |
|---|---|---|---|
| 10.0 | ++ | | +++ |
| 5.0 | ++++++ | | ++ - - |
| 2.5 | ++++ - | ++++ | |
| 1.0 | ++++ | ++++++++++++ | - - - - - - |
| 0.6 | ++++ | | - - - - |
| 0.4 | ++++++++++++ | | - - - - |
| 0.3 | +++ | | |
| 0.2 | ++++++ - - - - | ++++ - - - - - | |
| 0.1 | ++++ - - | ++ - - | |
| 0.08 | ++++ - - | | |
| 0.04 | + - - - - | - - - - - - - - | |

+ Indicating removal of less than 1/3 of epithelium (protection).
− Indicating removal of more than 2/3 of epithelium (non-protection).
Each sign stands for one cornea tested. Amount of histamine injected per cornea: 2 $\mu$g.

than 1/3 with + and absence of protection with removal of more than 2/3 of the epithelium with —. It can be seen that pyribenzamine and N-(2-pyridyl)-N-(2-thenyl)-N′, N′-dimethylethylenediamine hydrochloride* provide protection in concentrations of less than 1/10 of that of histamine while the protection by Antistine requires a concentration of 2-5 times that of histamine.

* This compound is commercially designated as 01013 and is listed in the table by the initials PTDA.

*Summary.* The loosening of the upper layers of the epithelium of the excised bovine cornea by histamine is prevented by simultaneous injection of certain antihistamines. Three compounds found to be effective are Pyribenzamine, 01013, and Antistine.

We are indebted for the supply of the antihistamines to Dr. B. N. Craver of the Ciba Pharmaceutical Products, Inc. (Pyribenzamine, Antistine), and to Dr. K. K. Chen of Eli Lilly Co. (01013).

## 16771

## Serum Polysaccharide Level in the Normal State.*

M. R. SHETLAR, JANEAL VILLET FOSTER, KEITH H. KELLY AND MARK R. EVERETT.
*From the Department of Biochemistry, University of Oklahoma School of Medicine, Oklahoma City, Okla.*

Several investigations concerning the polysaccharides of serum in both normal and pathological conditions have recently been published. Seibert and Atno,[1] and Seibert, Seibert, Atno, and Campbell[2] have presented results of quantitative studies using the carbazole method. Hinsberg and Merten[3] published results using an acid hydrolysis procedure while Lustig[4] *et al.* have reported several studies using an orcinol method. Nil-

* Aided by a grant from the Oklahoma Division of the American Cancer Society and from the John Archer Hatchett Memorial Fund.

1 Seibert, F. B., and Atno, A. J., *J. Biol. Chem.*, 1946, **163**, 511.

2 Seibert, F. B., Seibert, M. V., Atno, A. J., and Campbell, H. W., *J. Clin. Invest.*, 1947, **26**, 90.

3 Hinsberg, K., and Merten, R., *Z. Klin. Med.*, 1938, **135**, 76; *Brit. Chem. Physiol. Absts.*, 1939A, III, 357.

son[5] reported elevation of the glucosamine level of serum in pneumonia. His values for average normals were: for adults 77 mg % and for fetal serum 32 mg %; pneumonia serum averaged 183 mg %. West, Clarke, Kennedy[6] reported elevations in serum glucosamine in infections, disseminated malignant disease, and sterile infarcts.

As this laboratory is engaged in a study of the animal polysaccharides in relationship to malignancy, using a tryptophane method[7] for the determination of non-glucosamine serum polysaccharides it was necessary to investigate the normal polysaccharide pattern as well as that of as many pathological conditions as possible. It is the purpose of this paper to present the results of a complete study of normal subjects. The results of our study of pathological conditions will be published at a later date.

In order to give a more complete picture, non-glucosamine polysaccharide, glucosamine, tyrosine, and total protein determinations were made on ethanolic precipitates from the same serum.

*Experimental. Chemical methods.* Non-glucosamine polysaccharide was determined as described in a previous publication.[7] Total protein was determined, after precipitation of the serum protein with ethanol, by a micro kjeldahl procedure.

Preliminary work with the West, Clarke and Kennedy[6] method for determining glucosamine indicated that a considerable coloration could be developed with serum hydrolysates without the acetylacetone coupling reaction, consequently the method was modified as follows to give a blank for each hydrolysate.

*Reagents.* 1. Absolute Ethanol. 2. 8 *N* Hydrochloric Acid. 3. 2.5 *N* Sodium Hydroxide. 4. 2.5 *N* Hydrochloric Acid. 5. Acetylacetone Solution: 0.2 ml of acetylacetone dissolved in 10 ml of 0.5 *M* Sodium Carbonate (53 g $Na_2CO_3$ per liter of solution). This solution must be freshly prepared before using. 6. Ehrlichs reagent: Dissolve 5 g of p-dimethylaminobenzaldehyde in 190 ml of absolute ethanol and then add 190 ml of concentrated hydrochloric acid.

*Procedure.* Add 1 ml of serum drop by drop to 18 ml of absolute ethanol contained in a 15 x 150 mm pyrex test tube. Allow to stand 5 minutes and centrifuge. Pour off the supernatant liquid and invert the tube to drain the excess alcohol from the precipitate. Add 2 ml of water to the precipitate and stir with a glass rod. Add 2 ml of 8 *N* HCl and hydrolyse the mixture in a boiling water bath under reflux for 4-5 hours.

Filter through Whatman No. 1 filter paper into 10 ml volumetric flasks. Make up to volume and mix. Pipet 1 to 2 ml of the solution into three 15 ml calibrated centrifuge tubes, add a drop of phenolphthalein indicator to each and carefully neutralize with 2.5 *N* sodium hydroxide. Make slightly acid by adding a drop of 2.5 *N* hydrochloric acid. Add water to bring total volume to 3.5 ml. Add 2 ml of the acetylacetone solution to 2 of the tubes and 2 ml of 0.5 *M* $Na_2CO_3$ to the third, which serves as a blank. Stopper lightly and place in a boiling water bath for 20 minutes.

Remove from bath and cool below 37.5°C. Make volume to 10 ml with absolute ethanol. Add exactly 2 ml of Ehrlichs solution and mix. Stopper lightly and incubate in 37.5°C oven for 45 minutes. Determine optical density in Coleman 11 Spectrophotometer at a wave length of 540 mμ. Read each sample against its repective blank. A standard containing 100 μg of glucosamine-HCl (83.4 μg glucosamine) is subjected to the same procedure with each set of samples.

*Calculations*

$$\frac{Kx \times 83.4 \times 10}{Ks \times V} = \mu g \text{ of glucosamine per ml of serum}$$

Kx = optical density of sample

Ks = optical density of standard

4 Lustig, B., and Langer, A., *Biochem. Z.*, 1931, **242**, 321; Lustig, B., and Nassau, E., *Am. Rev. Tuberculosis*, 1941, **43**, 817; Novak, J., and Lustig, B., *J. Mount Sinai Hosp.*, 1947, **14**, 534.

5 Nilsson, I., *Biocem. Z.*, 1937, **291**, 254.

6 West, R., Clarke, D. H., and Kennedy, E. M., *J. Clin. Invest.*, 1938, **17**, 173.

7 Shetlar, M. R., Foster, J. V., and Everett, M. R., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 125.

TABLE I.
Summary of the Non-glucosamine Polysaccharide, Glucosamine, Total Protein and Tyrosine Content of Normal Serum.

| Group | No. | Polysaccharide, mg % | Polysaccharide, as % of protein | Glucosamine, mg % | Total protein, % |
|---|---|---|---|---|---|
| Fetal | 15 | 80 (62-103) | 1.41 (1.05-1.77) | 48 (42-55) | 5.68 (4.26-6.76) |
| Children (3-8 yrs) | 8 | 105 (94-118) | 1.60 (1.47-1.82) | 63 (52-69) | 6.59 (5.40-6.48) |
| Young adults: | | | | | |
| Males (21-32 yrs) | 18 | 110 (93-126) | 1.58 (1.26-2.02) | 66 (62-78) | 6.98 (6.00-7.48) |
| Females (22-49 yrs) | 10 | 111 (100-125) | 1.58 (1.42-1.81) | 68 (61-78) | 7.01 (6.26-7.35) |
| Aged (61-85 yrs) | 15 | 129 (104-138) | 1.79 (1.62-2.06) | 81 (70-89) | 7.20 (7.09-7.68) |

(Figures in parenthesis indicate the high and low figures in each group.)

V = volume of aliquot of hydrolysate

*Selection of Subjects.* A group of young adults was selected at random from volunteer medical students who had no recent history of any pathological condition. The children's group was obtained from a local orphanage. The aged group comprised selected subjects from the outpatient clinic who had only psychosomatic complaints or fully matured cataracts, or patients selected from a State Mental Hospital. The latter patients were selected with the aid of the institutional staff who were familiar with the condition of each patient through observation over a considerable period of time. These patients were in excellent physical condition aside from mental deterioration. The fetal blood samples were drawn by needle from the umbilical vein of the cord on the placental side immediately after clamping and cutting the cord.

*Results.* As it is impossible to include the actual analyses of all cases, a summary of normals is shown in Table I. The original data were treated to statistical analysis, in which the averages of the different groups were compared by the following formula from Rider.[8]

$$t = \frac{X_1 - X_2}{\left(\frac{N_1 + N_2}{N + N - 2}\right)^{1/2} \left(\frac{N_1 S_1^2 + N_2 S_2^2}{N_1 N_2}\right)^{1/2}}$$

$X_1$ and $X_2$ are the averages of the respective groups, $N_1$ and $N_2$ are the number of individuals in the respective groups, and $S_1^2$ and $S_2^2$ are the variances of the respective groups. This comparison is summarized in Table II.

From the data it appears that both the polysaccharide and glucosamine associated with serum protein tends to increase with age. Levels of fetal individuals are significantly lower than those of young adults, while levels of aged persons are significantly elevated. The levels for children were found to be lower than those for young adults, but the differences were not striking. The difference is not entirely proportional to levels of the serum protein in the various groups, although fetal serums were significantly lower in protein content. When the polysaccharide content for each sample is divided by the corresponding total protein, and the data treated to statistical comparison, the "t" value for the fetal group as compared to the young adult group is 2.669, a figure which is significant at the 2% level.

Since the interrelationships between the different components are important, total protein, non-glucosamine polysaccharide, and glucosamine levels of sera from the young adults were tested for correlation. The multiple correlation coefficient for these three factors was found to be 0.601, a value which is significant at the 1% level ("t" value = 3.76). The partial correlation between protein and polysaccharide independent of glucosamine, was found to be 0.576, and the partial correlation between polysaccharide and glucosamine, independent of total protein, was found to be 0.658. Both of these figures are significant at the 1% level. The correlation between glucosamine and total protein, independent of non-glucosamine polysaccharide was found to be 0.251, a figure which failed to be significant. The reason for this lack of correlation is not immediately apparent.

If all the polysaccharides of serum are composed of equimolecular proportions of man-

[8] Rider, P. R., Modern Statistical Methods, John Wiley & Sons, New York.

TABLE II.
Statistical Comparison of Results on Normal Subjects.

| Constituent | Groups compared | Difference % | t | D.F. |
|---|---|---|---|---|
| Total protein | Male v. female | 0.03 | 1.280 | 26 |
| Polysaccharide | " " " | 1.13 mg | 0.066 | 26 |
| Glucosamine | " " " | 1.56 " | 0.648 | 26 |
| Total protein | Young adults v. fetal | 1.31 | 7.941* | 41 |
| Polysaccharide | " " " " | 30.37 " | 9.230* | 41 |
| Glucosamine | " " " " | 19.13 " | 12.003* | 41 |
| Total protein | Young adults v. children | 0.40 | 2.185† | 34 |
| Polysaccharide | " " " " | 5.45 " | 1.445 | 34 |
| Glucosamine | " " " " | 3.75 " | 1.797 | 34 |
| Total protein | Young adults v. aged | — 0.22 | 1.707 | 41 |
| Polysaccharide | " " " " | —18.22 " | 6.107* | 41 |
| Glucosamine | " " " " | —13.53 " | 7.517* | 41 |

* Significant at the 1% level.
† Significant at the 5% level.

nose, galactose, and glucosamine, the glucosamine content divided by the non-glucosamine polysaccharide should be 0.498. The actual average figures were found to be as follows: Fetal 0.593, children 0.598, young adults 0.609, aged 0.626, weighted average 0.607.

The probability that the difference between the actual figure (0.607) and the theoretical value (0.498) could have arisen by chance is only 2.33 in 100, consequently it appears that all serum polysaccharides are not polymers of galactose-mannose-glucosamine in equimolecular proportions. There also appears to be a tendency for the ratio to increase with age, however this increase was not significant on the basis of the data obtained in this study.

*Variation from time to time.* In order to check the variability of serum polysaccharide in an individual, from time to time, samples were secured periodically over a period of 16 months. During this period the non-glucosamine polysaccharide varied between 110 and 122 mg %, the glucosamine between 70 and 78 mg % and the protein between 7.20 and 7.95%. This variation in polysaccharide is somewhat smaller than that recorded by Seibert and Atno,[1] and suggests that the polysaccharide level in healthy individuals is subject to about the same variation as the serum protein.

*Effect of Diet.* No significant difference was found in the polysaccharide levels determined on the same person fasting, 1 hour after a large meal, or 4 hours after the same meal (non-glucosamine polysaccharide levels of 115, 122 and 120 mg % respectively). Oral administration of 25 g mannose, 100 g galactose, or 50 g glucosamine caused no significant change in the polysaccharide level after 3 hours, 24 hours, or 72 hours. The polysaccharide levels were not appreciably affected by fasting (for 31 and 55 hours), by a high protein diet, by a low protein diet, or by high water intake (for 33 hours).

*Stability of serum polysaccharide during storage.* Data obtained from serum kept in the refrigerator for various lengths of time are recorded in the following table:

| Days stored | Polysaccharide | T. protein | Glucosamine |
|---|---|---|---|
| 0 | 120 | 7.20 | 75 |
| 1 | 117 | — | 73 |
| 2 | 115 | — | 73 |
| 3 | 114 | — | 76 |
| 7 | 115 | 7.32 | 74 |

*Effect of menstrual cycle.* Polysaccharide, glucosamine, and total protein determinations were made on the serum of 2 normal females at various times during the menstrual cycle (on the 2nd, 4th, 14th, 17th, and 22nd days of the cycle). Both subjects had normal 28 day cycles. The polysaccharide level varied between 111 and 118 mg % in one subject and 108 and 124 mg % in the other with no apparent correlation between the menstrual cycle and the polysaccharide level.

*Summary and conclusions.* Studies of serum polysaccharides were made on a total of 66

normal persons including fetal, children, young adult, and aged representatives.

The serum polysaccharide (both non-glucosamine and glucosamine) was lowest in the fetal group and highest in the aged representatives, the children and young adult groups being intermediate, thus showing a tendency to increase with age.

Concentrations of serum polysaccharide (both non-glucosamine and glucosamine) for young adults were significantly higher than those of fetal serums, and significantly lower than for the aged. The total protein for young adults was significantly higher than in fetal sera. Significant positive correlations were found between non-glucosamine polysaccharide and total protein and also between non-glucosamine polysaccharide and glucosamine.

In the same individual, non-glucosamine serum polysaccharide and glucosamine were subject to about the same variation as serum protein over a period of sixteen months. Small variations in diet appeared to have little effect. No appreciable changes occurred in serum polysaccharides during the menstrual cycle.

16772

## Failure of Trypan Red to Protect Against Certain Neurotropic Viruses ("M.M." and Russian Spring-Summer Encephalitis).*

W. McD. HAMMON, R. B. AIRD AND GLADYS SATHER.

*From the George Williams Hooper Foundation for Medical Research, and the Division of Neurology of the University of California, San Francisco.*

Investigations by Aird[1] on brilliant vital red and by Aird and Strait[2] on trypan red have shown these supra-vital dyes to affect the distribution of intravenously injected cocaine within the central nervous system. This was presumably due to an alteration of the permeability of the blood-brain barrier since the amount of cocaine reaching the cerebral cortex of cats receiving the dyes was decreased by 31% as compared with the controls not receiving the dyes. As a result of these studies and others, the question was raised as to the possible protective effect of these substances against infection of the central nervous system by neurotropic viruses. Due to the interruption of our studies by the late war we were unable to engage in experimental work along these lines.

Wood and Rusoff[3] claimed to demonstrate the protective effect of trypan red on the "M. M." virus.[4] Unfortunately, Wood and Rusoff inoculated the dye repeatedly by the intraperitoneal route and subsequently inoculated the virus by the same route. It has been the general experience of bacteriologists and immunologists that a local, non-specific protection against most infectious agents is set up in the peritoneal cavity by many types of inert substances when given previously by the same route.

Further studies on the possible protective effect of the supra-vital dyes against virus infections of the central nervous system, therefore, appeared indicated. Considering the mode of action of the dyes, it seemed wise to select viruses capable of infecting the central nervous system when inoculated by a peripheral route, as opposed to viruses requiring direct inoculation into the central

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc., and the Christine Breon Fund.

1 Aird, R. B., *Arch. Neurol. and Psychiat.*, 1939, **42**, 700.

2 Aird, R. B., and Strait, L., *Arch. Neurol. and Psychiat.*, 1944, **51**, 54.

3 Wood, H. G., and Rusoff, I. I., *J. Exp. Med.*, 1945, **82**, 297.

4 Jungeblut, C. W., and Dalldorf, G., *Am. J. Pub. Health*, 1943. **33**, 169.

TABLE I.
Effect of Trypan Red on Russian Spring-Summer Encephalitis Virus in Mice.

| Date of experiment | No. of mice | $LD_{50}$ controls | $LD_{50}$ trypan red | $LD_{50}$ Difference |
|---|---|---|---|---|
| 8/27/46 | 60 | 5.75 | 5.75 | 0.00 |
| 1/20/47 | 78 | 5.76 | 6.44 | +0.68 |

TABLE II.
Effect of Trypan Red on "M.M." Virus in Mice.

| Date of experiment | No. of mice | $LD_{50}$ controls | $LD_{50}$ trypan red | $LD_{50}$ Difference |
|---|---|---|---|---|
| 5/23/47 | 47 | 5.63 | 5.52 | —0.11 |
| 23 | 47 | 6.42 | 6.38 | —0.04 |
| 7/16 | 80 | 6.08 | 5.68 | —0.40 |
| 10/25 | 189 | 6.48 | 6.33 | —0.15 |

nervous system. Our experiments were, therefore, planned to employ the same virus used by Wood and Rusoff, and in addition the Russian spring-summer encephalitis virus (Sophy strain-eastern type).† Both of the viruses infect readily by the intraperitoneal and other peripheral routes. The route or mode of invasion of the central nervous system, however, is as yet unknown for both viruses. It is entirely possible that they are not carried by the blood stream (as is cocaine) but may invade through peripheral nerves.

Mice were inoculated repeatedly as indicated below, with 1% trypan red in saline by the subcutaneous route over a period of several days. This resulted in their skin assuming a bright reddish tint. Controls were given saline in the same dosage. Following this preparation all mice were inoculated with one or the other of the viruses by the *intraperitoneal* route. Serial dilutions of the virus were prepared from frozen ampoules and four 10-fold dilutions were selected to cover the range of virus from that which would kill all mice to that which would not kill any. The LD-50 was calculated by the method of Reed and Muench.[5]

*Russian Spring-summer virus.* The mice were prepared by 7 subcutaneous inoculations of 0.1 to .05 cc of 1% trypan red or with saline over a period of 4 weeks. Five days after the last injection the virus was inoculated intraperitoneally. The results of 2 consecutive experiments employing 138 mice are shown in Table I. It will be noted that, if any effect was produced, the mice inoculated with the dye were the more susceptible. In our opinion, however, the difference shown is not significant.

*"M. M." virus.* In these experiments less dye was given. The dosage given by Wood and Rusoff was followed exactly: 0.1 cc of a 1% solution being injected on 3 successive days. The virus was given one day after the last dye injection. As in all our tests the dye was given subcutaneously and the virus intraperitoneally. The results of 4 successive experiments employing 363 mice, are shown in Table II. It may be observed that the differences between the controls and those inoculated with dye ranged from 0.11 to 0.40 log and never attained a significant difference. However, in each experiment the small differences which did appear always occurred in the same direction; a lower LD-50 in those receiving the dye. It was felt that this effect, however, could well be due to chance alone.

*Conclusions.* Trypan red failed to produce a significant protection against Russian Spring-summer encephalitis virus and "M.M." virus when the dye was injected subcutaneously and the virus was inoculated by the intraperitoneal route. These experiments fail to confirm the results of others who injected both dye and virus intraperitoneally. We in-

† Obtained through the courtesy of the Army Medical Department Virus and Rickettsia Laboratories.

5 Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

terpret the apparent protective effect obtained by Wood and Rusoff[3] as being due to a well recognized and non-specific peritoneal protection afforded by certain inert substances when injected previous to the inoculation of infectious agents by the same route. Our results suggest that these two neurotropic viruses do not enter the central nervous system directly by way of the vascular channels as is the case with such an agent as cocaine, the passage of which into the central nervous system tissue may be altered by trypan red.

We wish to acknowledge the technical assistance of Doctor David Zealear and Miss Sylvia Bowditch in one phase of this study.

16773

## Unidentified Growth Factor(s) Needed for Optimum Growth of Newborn Pigs.

A. L. Neumann, J. L. Krider and B. Connor Johnson

*From the Department of Animal Science and the Division of Animal Nutrition, University of Illinois, Urbana.*

An unidentified growth factor(s) (the animal-protein factor, factor X,[1] vitamin $B_{12}$,[2] vitamin $B_{13}$,[3] etc.) has been reported to be present in certain concentrates such as anti-pernicious anemia liver extract. It has been reported that this factor(s) will improve the growth rate of the rat,[4] the dog,[5] the fox,[6] and the chick.[7]

Since it has been shown[8,9] that newborn pigs can be raised successfully to weaning age on a synthetic ration made up to simulate milk, this appeared to be a suitable technic for determining whether the pig requires such an identified growth factor(s). Carey *et al.*[1] have reported that casein supplies significant amounts of factor X to the rat, and for this reason the "vitamin-free" casein used in previous studies[8,9] was replaced in the first experiment by an isolated soybean protein (alpha-protein).* Methionine was added to the diet, since this alpha-protein is deficient in sulfur amino acids.[10] The composition of the rations fed is given in Table I.

*Experimental.* Experiment I. Seven 2-day-old Duroc Jersey-Chester White cross-bred pigs from the same litter were divided into 3 groups on the basis of weight and age. The pigs were housed in individual raised wire cages in a heated building and were fed the following diets *ad libitum*. Group 1 received the casein "synthetic milk" ration[9] (Diet A, Table I); Group 2 received the alpha-protein "synthetic milk" (Diet B); and Group 3 received Diet B plus Reticulogen,† a 20-unit/ml anti-pernicious anemia liver extract. Individual feed consumption records were

1 Cary, C. A., Hartman, A. M., Dryden, L. P., and Likely, G. D., *Fed. Proc.*, 1946, **5**, 128.

2 Rickes, E. L., Brink, N. G., Koniusky, F. R., Wood, T. R., and Folkers, K. F., *Science*, 1948, **107**, 396.

3 Novak, A. F., and Hauge, S. M., *J. Biol. Chem.*, 1948, **174**, 647.

4 Jaffe, W. G., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **169**, 287.

5 Ruegamer, W. R., Torbet, N., and Elvehjem, C. A., *Fed. Proc.*, 1947, **6**, 187.

6 Schaefer, A. E., Whitehair, C. K., and Elvehjem, C. A., *Fed. Proc.*, 1947, **6**, 420.

7 Novak, A. F., Hauge, S. M., and Carrick, C. W., *Poultry Sci.*, 1947, **26**, 604.

8 Johnson, B. C., James, M. F., and Krider, J. L., *J. Animal Sci.*, 1947, **6**, 486.

9 *Ibid.*, *J. Animal Sci.*, 1948, **7**, 486.

10 Grau, C. R., and Almquist, H. J., *J. Nutrition*, 1943, **26**, 631.

* The alpha-protein was generously supplied by the Glidden Co., Chicago, Ill., through the courtesy of Dr. J. L. Gabby.

† The reticulogen (20-unit anti-pernicious anemia liver extract) was generously supplied by Eli Lilly and Co., Indianapolis, Ind., through the courtesy of Dr. E. C. Kleiderer.

TABLE I.
Composition of "Synthetic Milk" Rations.

| | Diet A Casein diet (Group 1) | Diet B Alpha protein diet (Group 2 and 3) | |
|---|---|---|---|
| Casein (Labco, vitamin-free) | 30 | | |
| Alpha protein (isolated soybean protein) | — | 29.7 | |
| Methionine* | — | 0.3 | |
| Glucose (cerelose) | 37.4 | 37.4 | |
| Mineral salts† | 6.0 | 6.0 | |
| Lard | 26.6 | 26.6 | |
| Reticulogen | — | 0.33 | ml/pig/day Group 3, Expt. I |

Made up and homogenized into a milk containing 13% solids including 4% lard (liquid basis).

The following vitamins‡ were added per 1000 g of milk:

| | | | |
|---|---|---|---|
| Thiamine | 0.65 mg | *p*-aminobenzoic acid | 2.6 mg |
| Riboflavin | 1.30 " | Pteroylglutamic acid | 0.052 " |
| Pyridoxine | 1.30 " | Biotin | 0.01 " |
| Calcium pantothenate | 7.8 " | α-tocopherol acetate | 1.0 " |
| Inositol | 26.0 " | 2-methyl-1,4 naphthoquinone | 0.26 " |
| Choline | 260.0 " | Vit. A | 1000 I.U. |
| Nicotinic acid | 2.67 " | Vit. D | 100 " |

* The *dl*-methionine was furnished by E. I. DuPont de Nemours and Company, Inc., New Brunswick, N.J.

† See Johnson *et al.*[9]

‡ The thiamine chloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, nicotinic acid, biotin, and α-tocopherol acetate used in this experiment were generously supplied by Hoffman-La Roche, Inc., Nutley, N. J. Pteroylglutamic acid was supplied by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y. Inositol was donated by A. E. Staley Manufacturing Co., Decatur, Ill. Hyflavin (a highly water-soluble riboflavin) was furnished by Endo Products, Inc., New York City.

TABLE II.
Results of Experiment I.

| | Groups and treatments | | |
|---|---|---|---|
| Items compared | I Casein milk | II Isolated soybean protein milk plus DL-methionine | III Same as II plus reticulogen |
| No. of pigs started | 2 | 2 | 3 |
| Av initial wt, g | 1780 | 1785 | 1523 |
| " wt at 21 days, g | 6940 | 6125 | 5600 |
| " final wt, g | 14610 | 13530 | 19960 |
| " daily gain during test, g | 231.5 | 209.8 | 326.9 |
| " " " to 21 days, g | 245.2 | 206.6 | 195.2 |
| " " " 21 to 56 days, g | 222.5 | 211.6 | 404.1 |

kept and the pigs were weighed at weekly intervals.

Reticulogen was given to the pigs in Group 3 at the rate of 0.33 ml per pig daily. To reduce intestinal synthesis of unidentified growth factors, sulfathaladine‡ was added to the diet at the rate of 2 g per liter of milk. The fat-soluble vitamins were added to the milk during homogenization, and the water-soluble vitamins were added to the milk at the time of feeding.

The gains as ratios of attained weight to initial weight for the 3 groups are plotted in Fig. 1, and the data are summarized in Table II. At 8 weeks the pigs in Group 3 (Reticulogen) had gained significantly more than those in Group 2 ($P = 0.007$)[11] or those in Groups 1 and 2 ($P < 0.001$).[11]

The photographs in Fig. 2 illustrate the

‡ Sulfathalidine (phthalylsulfathiazole) was generously supplied by Sharp and Dohme, Philadelphia, Pa., through the courtesy of Dr. S. F. Sheidy.

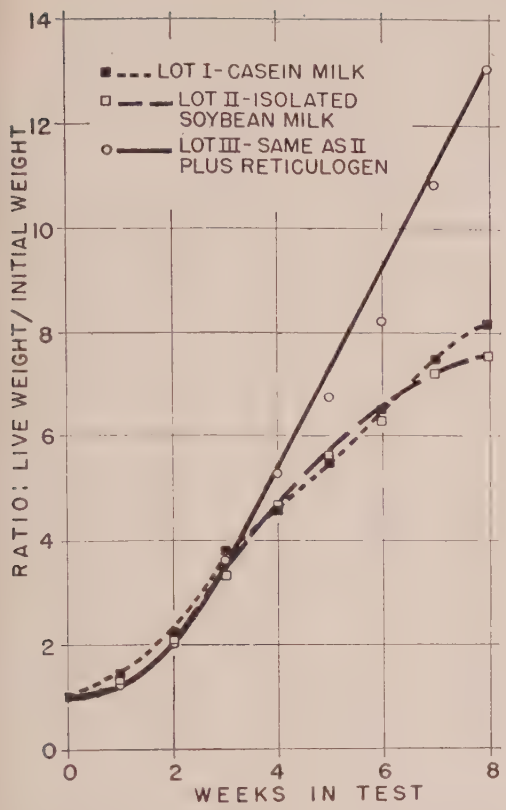



FIG. 1.
Average gains expressed as ratio of weight to initial weight.

improvement in thrift, size, condition and amount of haircoat, and soundness of feet and legs when Reticulogen was given as a source of the unidentified growth factor(s).

Normal hemoglobin levels and normal red and white cell counts were found in all pigs at 8 weeks.§

At the close of the experiment all pigs were placed together and given access to green pasture and a good farm ration for weanling pigs. After 3 weeks there had been practically no gain in the pigs from Groups 1 and 2, while those from Group 3 continued to gain normally. Only after 5 weeks on a good farm ration did the pigs on the unsupplemented rations overcome their growth factor(s) deficiency.

Experiment 2. In order to confirm the results of Experiment 1 in which only a small number of animals were used, a second experiment was carried out in which 21 two-day-old Duroc-Jersey pigs were used. In this experiment vitamin-free casein was used as the protein source in the ration, since the results of Experiment 1 had indicated that this vitamin-free casein was almost as deficient in the unknown factors present in Reticulogen as was alpha-protein.

The basal ration (Diet A, Table I) was fed to a group of 9 baby pigs. Another group of 12 baby pigs received the same ration plus 0.25 ml of Reticulogen per pig daily. The pigs were housed and fed as in Experiment 1.

Again, after the 8-week experimental period, the animals in the group receiving Reticulogen had gained significantly more than those on the basal ration ($P = <0.001$). These results are summarized in Table III.

11 Fisher, R. A., Statistical Methods for Research Workers, 9th Ed., Oliver and Boyd, Ltd., London, 1944.

§ We are indebted to Dr. Marian F. James for these determinations.

FIG. 2.
Representative pigs from the 3 lots. Left, Pig. No. 1 from the casein lot. Middle, Pig No. 3, from the alpha-protein lot. Right, Pig No. 6, from the alpha-protein + Reticulogen lot.

TABLE III.
Weight Gains in Experiment II.

| | Group I Basal ration A | Group II Basal ration A + reticulogen |
|---|---|---|
| No. animals | 9 | 12 |
| Av initial wt | 1.56 kg | 1.60 kg |
| Av final wt at 8 wks | 16.0 " | 21.6 " |
| Av gain in wt for 8 wks | 14.4 " | 20.0 " |

*Summary.* The addition of Reticulogen (a 20-unit anti-pernicious anemia liver extract) to a synthetic milk diet resulted in an increased growth rate in baby pigs over an 8-week period. The final weights of pigs receiving Reticulogen averaged 19.96 and 21.6 kg as compared with 13.53 and 16.0 kg for those not receiving Reticulogen in two comparisons.

16774

## Effect of a Progesterone Compound on Growth of a Transplanted Granulosa Cell Tumor.*

EUGENE E. CLIFTON† AND SHIH-CHENG PAN.‡ (Introduced by S. C. Harvey.)
*From the Departments of Anatomy and Surgery, Yale University School of Medicine.*

Progesterone has been considered to be inhibitory to, or without effect on, tumor growth. Heimann[1] reported a marked decrease in incidence of mammary carcinoma in the RIII strain of mice treated with progesterone alone and a still lower incidence when this was combined with testosterone. He stated, however, that it did not prevent growth of transplanted tumors in mice. He also found[1b] that it inhibited growth of adenomatous portions of breast fibroadenomata and decreased the number of takes of auto- and homo-transplants in rats. There was no effect on fibroma, myxoma, or sarcoma or on adenomatous growth in pregnant or castrated rats. In the guinea pig Lipschütz and Vargas[2] observed decrease in size, and actual disappearance, of the fibroids caused by oestrogen. Burrows and Hoch-Ligeti,[3] however, found no change in incidence of mammary carcinoma in mice injected weekly with 1 mg of progesterone, and Loesser[4] found slight, if any, improvement in animals or patients with breast carcinoma treated with progesterone.

The effects of larger amounts of progesterone on tumor growth have been studied, using a material containing a high concentration of progesterone.§ Preliminary experiments indicated a growth-stimulating effect on mammary carcinoma in mice.[5] A study of the effect of this material on a transplanted ovarian tumor was then undertaken. Subcutaneous transplantation of a granulosa cell tumor induced by intrasplenic transplants[6] in mice of the C57 strain had been attempted.

* This investigation was supported in part by grants from the Jane Coffin Childs Memorial Fund for Medical Research, and the United States Public Health Service Grant C343, administered by W. U. Gardner.

† Senior Fellow, American Cancer Society, as recommended by the National Research Council.

‡ Anna Fuller Fund Fellow. Present address: Hsiang-ya Medical College, Changsha, China.

1 a, Heimann, Jacob, *Cancer Research*, 1945, **5**, 426; b, Heimann, Jacob, *Cancer Research*, 1943, **3**, 65.

2 Lipschütz, A., and Vargas, L., *Lancet*, 1939, **2**, 420.

3 Burrows, Harold, and Hoch-Ligeti, Cornelia, *Cancer Research*, 1946, **6**, 608.

4 Loesser, A. A., *Lancet*, 1941, **2**, 698.

§ A crude preparation believed to contain 200-250 I.U. Progesterone/cc made available through the courtesy of E. A. Engstrom, The Glidden Co., Chicago, Ill.

5 Cliffton, E. E., unpublished data.

6 Li, M. H., and Gardner, W. U., *Cancer Research*, 1947, **7**, 549.

TABLE I.

| Treatment | Total No. animals | No. animals without growth | No. of animals with growth | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 1 wk | 2 wk | 3 wk | 4 wk | 5-7 wk | 8-12 wk | 12-16 wk |
| Glidden Progesterone | 26 | 4 | 1(1) | 11(10) | 20(9) | 22(2) | 22(0) | 22(0) | 22(0) |
| Oestradiol | 18 | 9 | 0 | 0 | 0 | 2(2) | 4(2) | 9(5) | — |
| Progesterone* | 8 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 1(1) |
| Control | 18 | 14 | 0 | 0 | 0 | 0 | 0 | 1(1) | 4(3) |

Incidence and time of apparent subcutaneous growth of tumor 18(57) with different hormone treatment.

The numbers in brackets are the numbers of animals showing first growth in that period while the unbracketed numbers are total tumor takes through that period.

*"Proluton" obtained through the courtesy of the Schering Corp., Bloomfield, N. J., containing 5 mg progesterone/cc.

TABLE II.

| Treatment | Total No. animals | No. animals showing growth in 6 wk |
|---|---|---|
| Glidden Progesterone 0.05 cc two times per week | 31 | 28 |
| Oestradiol 0.05 cc two times per week | 9 | 5 |
| Progesterone* ("Proluton") 0.1 cc two times per week | 4 | 0 |

Number of animals treated and showing growth of tumor within 6 weeks with 3 types of hormone treatment.

*"Proluton" obtained through the courtesy of the Schering Corp., Bloomfield, N. J., containing 5 mg progesterone/cc.

Growth of one of the transplanted tumors occurred but rarely and only after a long time in intact or castrated animals with and without estrogen treatment. In castrated animals treated with the crude progesterone, growth of the tumor occurred in some cases within 10 days. Subsequent study has confirmed this finding plus additional information.

*Methods.* Mice of the C57 strain approximately 2 to 3 months of age were castrated and a small piece of tumor was transplanted subcutaneously in each animal. The tumor was obtained from a first generation subcutaneous transplant. Injections were started on the day of transplantation. To facilitate injection, the crude material was diluted by adding 1 part Ethanol to 4 parts of the compound. The test animals in the first group received 0.05 ml of the diluted "progesterone compound" 3 times per week. Subsequent series received the diluted material 2 times per week. Control animals were set up in 4 groups: Group one received estradiolbenzoate (25 $\mu$g in 0.05 cc sesame oil 2 times a week); Group 2 received Ethanol (1 part to sesame oil 4 parts, 0.05 cc two times per week); Group 3 received progesterone‖ 0.05 cc daily, and subsequently 0.1 cc daily; and Group 4 received no injections. Groups 2 and 4 have been combined in the study of results (Tables I and II). All animals were kept on a standard diet and water *ad libitum,* with the same number of animals per cage. Alternate animals were used for each treatment.

Three series of animals so treated, confirmed the preliminary opinion that the crude, progesterone-containing compound, when injected into animals receiving a transplant of the granulosa cell tumor, stimulated growth of the tumor (Tables I and II). Seventy-seven per cent of the "progesterone compound" treated animals had growing tumor within 3 weeks, and 85% within 4 weeks, while in the other groups no palpable tumors were ob-

‖"Proluton" obtained through the courtesy of the Schering Corp., Bloomfield, N. J., containing 5 mg progesterone/cc.

served in the 3 week period and only 2 in the 4 week period. Allowing a period of at least 4 months for the control animals to develop tumors, it was found that 50% of the estrogen treated animals, 22% of the animals receiving Ethanol in sesame oil or no injections, and one, or 25% of the "proluton" treated animals showed tumor growth, to compare with the 85% tumor growth within 4 weeks with the test material.

One small series of intact mice with transplanted tumor, divided into "progesterone compound" treated, estrogen treated, "proluton" treated, and control groups of 3 animals each, was studied. These animals were also allowed to breed. Of this group only one, a progesterone compound treated mouse, developed a tumor.

Subsequent groups of animals with tumor transplants of the second and third generations, observed for only 6 weeks, are shown in Table II. These confirm the previous findings with 90% of the "compound" treated, 55% of the estrogen treated, and none of the "proluton" treated animals showing tumor growth.

Additional facts in evaluating the action of the "progesterone compound" are: (1) 7 of the "progesterone compound" treated animals of the original 26 died of their tumors within the period 1 to 3½ months after transplantation, while none of the other animals of the original 3 series died of their tumors up to 4 months after transplantation. Many animals were sacrificed in all groups but in every instance the animals in the control groups were permitted to live longer because their tumors were smaller; (2) 2 of the "progesterone compound" treated animals developed gross and several had microscopic metastases, while no animals of the other groups showed metastatic spread.

More detailed studies are being carried out. One obvious line of investigation will be the effect of other hormones especially androgens on the growth of this tumor. It is always possible that this growth stimulation is due to some other material present in the "compound", which can be proven only by more detailed chemical work.

## 16775 P

## Effects of Folic Acid on the Anemia Induced by X Irradiation.

S. PHYLLIS STEARNER. (Introduced by A. M. Brues.)

*From the Biology Division of the Argonne National Laboratory, Chicago, Ill.*

Previous work in this laboratory has shown that the anemia which followed radiations from internal and external sources was frequently macrocytic.[1,2] The macrocytosis may have been the result of damage directly to the bone marrow or indirectly through damage to the viscera, resulting in failure to form an antianemia factor. Folic acid, classified as a vitamin of the B complex, is reported to be similar in its action to liver extract. It has been shown to be effective therapeutically in certain of the macrocytic anemias in man.[3,4] It was without effect, however, upon the macrocytic anemia that resulted from administration of $Sr^{89}$.[5,6] Total-body X irradiation, unlike radioactive strontium, causes damage to the viscera as well as bone marrow. It is

[1] Stearner, S. P., Simmons, E. L., and Jacobson, L. O., to be published, 1948.

[2] Simmons, E. L., and Jacobson, L. O., Radiotoxicity of injected $Sr^{89}$ for rats, mice and rabbits. Part IV. The hematological effects of enterally and parenterally administered $Sr^{89}$ in mammals. National Nuclear Energy Series, Vol. 22b.

[3] Spies, T. D., Vilter, R. W., Koch, M. B., and Caldwell, M. H., *Southern Med. J.*, 1945, **38**, 707.

[4] Moore, C. V., Vilter, R. W., Manneck, V., and Spies, T. D., *J. Lab. and Clin. Med.*, 1944, **29**, 1226.

[5] Jacobson, L. O., Stearner, S. P., and Simmons, E. L., to be published.

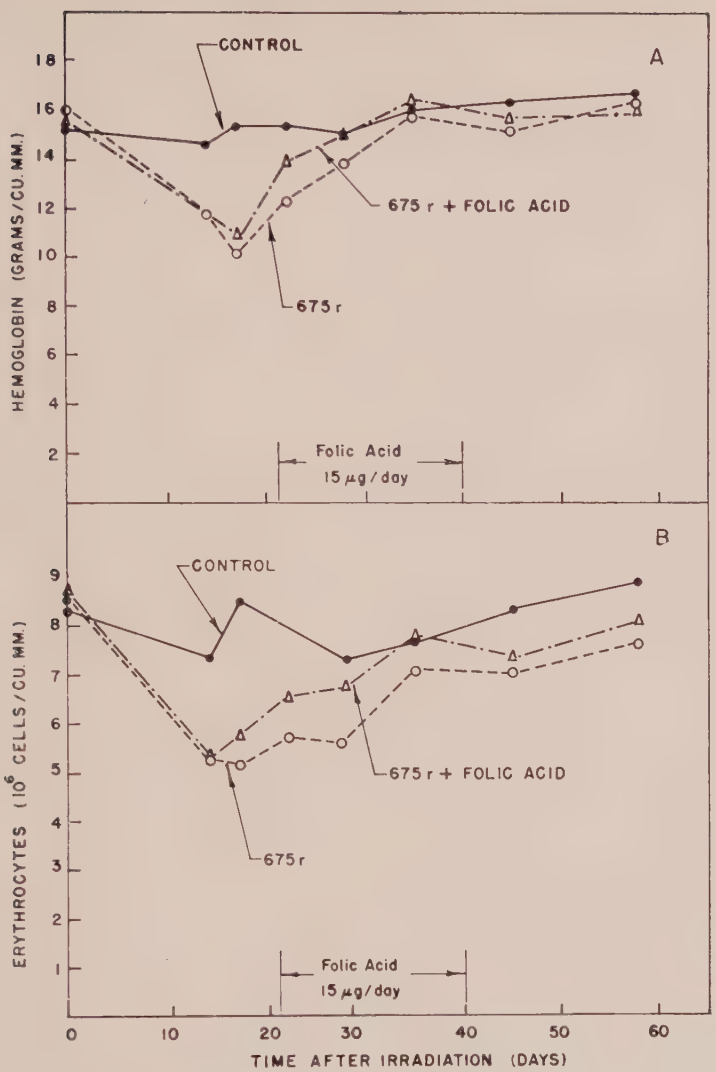



FIG. 1.

Effect of 675 r X irradiation and administration of folic acid on amount of hemoglobin (A) and number of erythrocytes (B) in the peripheral blood.

of interest, therefore, to determine the possible therapeutic effect of folic acid on the macrocytic anemia resulting from total-body X irradiation.

Male white rats were irradiated with 675 r (the approximate median lethal dose). Conditions of irradiation were: 200 Kv, 15 ma, 0.5 mm Cu and 1.0 mm Al filters, target field distance 72.5 cm, and exposure rate about 16 r per minute. One half of the irradiated animals were given intraperitoneal injections of folic acid daily, 5 days per week. The animals were divided into 3 groups and treated as follows:

| Group | No. | Treatment |
|---|---|---|
| 1 | 10 (5 blood animals) | Controls |
| 2 | 10 (5 blood animals) | 675 r |
| 3 | 10 (5 blood animals) | 675 r, and receiving 15 μg folic acid per day for the period 22-40 days after irradiation |

[6] Jacobson, L. O., Stearner, P., and Simmons, E. L., *J. Lab. and Clin. Med.*, 1947, **32**, 1425, Abst. 37.

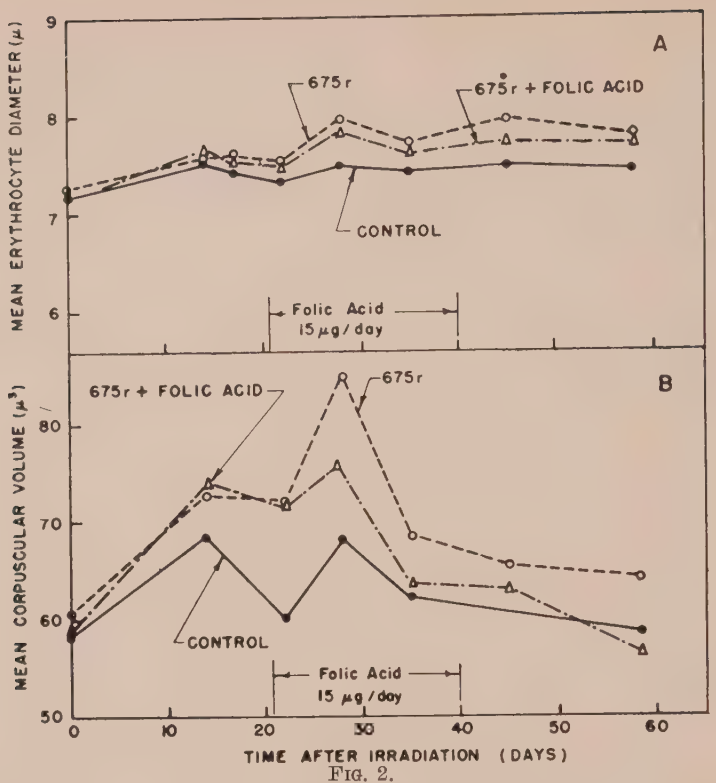



FIG. 2.

Effect of 675 r X irradiation and administration of folic acid on mean erythrocyte diameter (A) and mean corpuscular volume (B).

About 2 weeks after exposure the mean hemoglobin and erythrocyte values in the peripheral blood fell to about 70% of that of the control (Fig. 1). At most sampling intervals the irradiated group that received folic acid showed slightly higher values than the untreated irradiated group. The mean erythrocyte diameter was increased in the irradiated groups at 3 weeks after exposure and persisted for the duration of the observation. Fig. 2 shows the macrocytosis was approximately equal in the 2 irradiated groups and corresponded to the increase in mean corpuscular volume. However, the increase in mean erythrocyte volume was slightly greater in the untreated than in the folic acid treated irradiated group. The greatest increase in per cent of reticulocytes occurred before the maximum mean corpuscular volume was reached (Fig. 3) and therefore could not have caused the increase in mean erythrocyte diameter and volume. The administration of folic acid had no apparent effect on the reticulocyte response following irradiation.

Analysis of variance[7] between Group 2 and Group 3, for all sampling periods from 22 to 58 days (inclusive) after irradiation, indicated a significant difference ($P \geqq 0.01$) in only the mean erythrocyte count and mean corpuscular volume. (The variance introduced as a result of separate group caging does not affect the statistical results and, for simplicity of presentation, was ignored). The importance of this difference is minimized because the related factors (hemoglobin and mean erythrocyte diameter) did not show a significant difference. It appears, therefore, that there is little or no effect of folic acid upon the erythropoietic system following exposure to X radiation. The damage to the

[7] Tyler, S., personal communication.

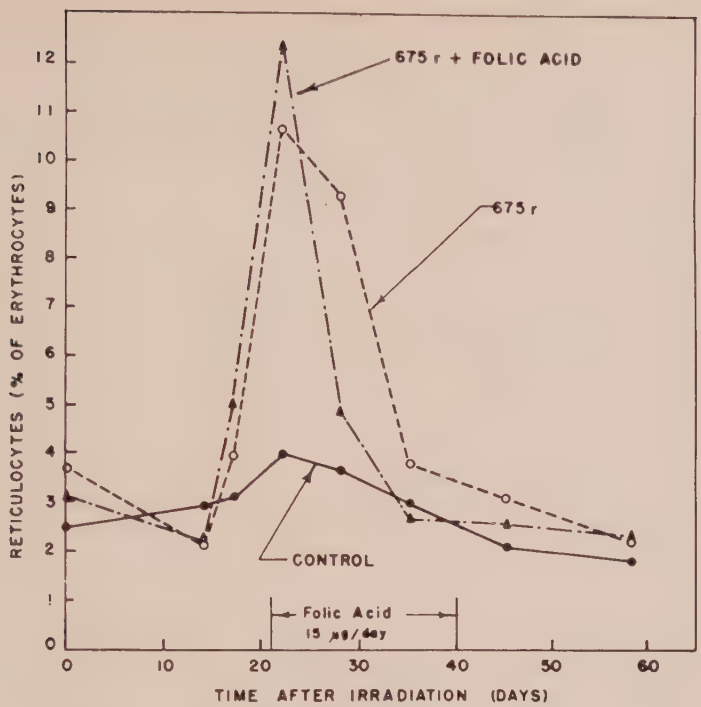



FIG. 3.

Effect of 675 r X irradiation and administration of folic acid on % of reticulocytes in the peripheral blood.

bone marrow is probably caused principally by direct injury. Any indirect damage resulting from damage to the viscera, which produces the antianemia principle, must be a relatively minor source of injury.

16776

## Genetic Changes in Gastric Lesion and Fibrosarcoma Susceptibilities.*

LEONELL C. STRONG.

*From the Department of Anatomy, Yale University School of Medicine.*

Evidence has been published that the gene for brown hair has mutated to dominant black several times in the descendants of mice which had been injected with methylcholanthrene for several generations. Concomitant with the production of these dominant hair color mutations there were also obtained (1) a considerable increase in susceptibility to induced fibrosarcomas, (2) an increased litter size, and (3) increased vitality. All 3 of these characteristics appear to be associated with the black mutant character, never with their brown litter mates. The evidence points toward the production of a widespread germinal change brought about by the effect of methylcholanthrene upon the germ plasm,

* This experiment has been made possible by grants from The Anna Fuller Fund, The Jane Coffin Childs Memorial Fund for Medical Research, and The Committee on Growth of the National Research Council acting for The American Cancer Society.

but a point mutation at a single locus has not as yet been completely excluded.[1]

It has also been demonstrated that the gene for susceptibility to induced fibrosarcomas in mice shows linkage relationship with the gene for black hair pigmentation. The essential data of crossing over between these two genes on the brown tagged chromosome have already been obtained.[2]

A gene for susceptibility to a stomach lesion occurring primarily in the gastric mucosa of NHO mice also shows linkage relationship with the brown gene.[3]

The present paper reports the data obtained on the incidence of gastric lesions following the subcutaneous injection of methylcholanthrene in the black mutants and their litter mate brown segregates. Of the black mutants 6 descents have been continued for several generations, 3 of these descents were obtained from a brother-sister mating of two of the original black mutants, whereas the other 3 descents were descended from a backcross between one of the black mutants and its own mother of the original brown ancestry. Each of these 3 descents were made up of (a) pure breeding blacks, (b) derived pure breeding brown segregates, and (c) black heterozygous for brown. All mice were weaned at 30 days of age, mated together and injected subcutaneously in the right groin with 1 mg of methylcholanthrene dissolved in 0.1 cc of sesame oil at 60 days of age. They were examined periodically for the appearance of tumors and signs of sickness. All mice were killed when progressively growing tumors were evident or when they showed one or more signs of sickness, such as emaciation, wheezing or a ruffled appearance of the hair.

It is impossible to demonstrate whether these 3 black mutants which appeared in one litter of mice were derived from one original mutation or from 3 separate ones. If the mutation occurred in one germinal epithelial cell and then the mutant cell gave rise to 3 eggs, it is possible that one mutation was responsible for all 3 mutants. This interpretation is consistent with the embryology of the mouse. There are further considerations that only one mutation was involved. (1) It is highly improbable that any inductive system no matter how specific in action would produce three mutations at the same locus in 3 separate animals without inducing other mutations at other loci. This did not occur. (2) The 3 mutants are the same as far as the somatic manifestation of the mutant gene is concerned. They are each characterized by a peculiar distribution of pigment granules in the hairs which simulates but differs somewhat from the pigment distribution in the well known black mutation, as found in mice of the $C_{57}$ black stock.[3] All 3 black mutants gave comparable Mendelian ratios when crossed with each other or in the $F_2$ and backcross generations to the recessive condition, brown, following an outcross to mice of the Strong A strain. For these reasons it is considered that the three mutants are genetically and biologically similar if not identical and the data obtained on the descendants of all these can be legitimately classified together.

Of these mice derived from the black mutants and injected with methylcholanthrene, 362 developed lesions involving the stomach, as follows: 198 black to 164 browns. These gastric lesions consisted of several histological types, comparable to the varieties already obtained with methylcholanthrene in the original brown ancestry. There were 3 distinct regions of the stomach of the mouse which gave rise to neoplasia, as follows: (1) a squamous type from the fore-stomach, (2) mixed types from the region of the limiting ridge, and (3) the lesions just anterior to the pylorus involving the mucus secreting neck cells of the gastric mucosa. In the black series the sex differential of gastric lesions was 1.95 males to 1.00 females, whereas in the brown series the sex differential was 2.40 males to 1.00 females. The distributions of gastric lesions for the two color classes of mice are given in Chart 1: brown mice on the short dash line, black mice on the solid line.

[1] Strong, L. C., *Yale J. Biol. and Med.*, 1946, **18**, 359.

[2] Strong, L. C., *Science*, 1946, **103**, 554.

[3] Strong, L. C., *J. Nat. Cancer Inst.*, 1945, **5**, 339.

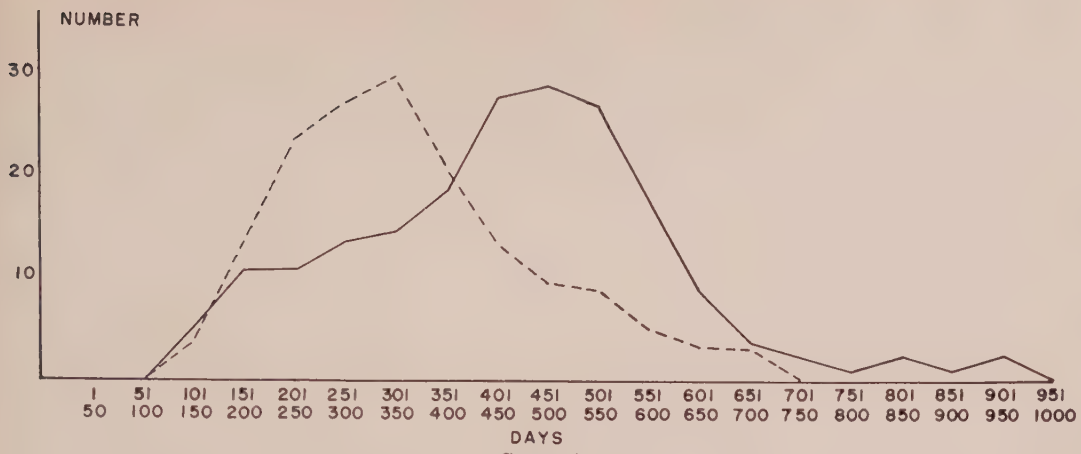



CHART 1.

This chart presents the data on the frequency distribution of induced gastric lesions in mice of the Bl subline; time in days is given on the base line, the number of mice on the vertical line. Black mutants are given on the solid line, brown segregates on the short dash line.

The average latent period for the appearance of the gastric lesions in black mice was 434.4 days, whereas the average latent period for the brown mice was 335.8 days. This is a difference between the two color classes of 98.6 days. The average latent period of gastric lesions in the original brown descent from which the black mutation had occurred was 318.5 days. Thus it is evident that the brown segregates from the black mutants retain the same latent period for gastric lesions as their brown ancestors (335.8 days-318.5 days = 17.3 days difference) whereas the black mutants have acquired a delayed latent period of 115.9 days beyond the original brown ancestry (434.4 days - 318.5 days = 115.9 days).

The present data on the incidence of tumors induced in black mutants by the subcutaneous injection of methylcholanthrene has therefore, led to the conclusion that while one type of tumor susceptibility (fibrosarcomas at the site of injection) is being increased, another type, the gastric lesion, is being decreased. This is evidence that in this particular experimental setup the two cancer susceptibilities are distinct entities even though they both show linkage relationships with the brown tagged chromosome. The exact spatial relationship of the 3 entities or genes on the brown chromosome cannot at present be determined. Another conclusion that seems justified is that in the origin of the original black mutant more than a single point mutation was involved. This finding is of increased significance, since, as far as can be determined now, the original mutation from brown to black itself was a clear cut point mutation, as no variations from typical Mendelian ratios could be determined in the inheritance of mutant black. Skewness to the right of the frequency distribution curve for gastric lesions in brown mice and to the left for the black mutants is suggestive that this may be brought about, in part, by the two cross-over types, a brown mouse with a delayed latent period for a gastric lesion and a black mouse with an enhanced or original early appearance of the gastric lesion. Unfortunately no progeny from these mice were obtained so that this genetic evidence of crossing over could not be obtained.

The present evidence also indicates that

TABLE I.

| | Bl descent | | |
|---|---|---|---|
| | Black | Brown | Total |
| Gastric lesions | 198 | 164 | 362 |
| Normal | 2145 | 1452 | 3597 |
| Total | 2343 | 1616 | 3959 |

This table presents data on mice of the Bl descent injected with methylcholanthrene at 60 days of age. The mice are classified according to whether they developed gastric lesions or did not develop such a lesion. The mice are further divided into the two color classes, as follows (a) the original color class ,brown and (b) the mutant black.

there are specific entities or genes underlying genetic susceptibilities to specific types of neoplasia induced by methylcholanthrene. This interpretation seems to be favored over the alternative concept that in cancer susceptibility, there is a general cancer "gene" that underlies all types of cancer. That is, that there is a gene that determines the difference between the biological states of cancer and not cancer.

16777

## Mucolytic Enzyme Systems. IV. Relationship of Hyaluronidase Inhibition by Blood Serum to Incidence of Mammary Cancer in Mice.*

DAVID GLICK, THOMAS A. GOOD AND JOHN J. BITTNER.

*From the Departments of Physiological Chemistry and Cancer Biology, University of Minnesota Medical School, Minneapolis, Minn.*

From previous investigations in this series, it was concluded that the level of hyaluronidase inhibitor in blood serum is elevated in a wide variety of both virus and bacterial infections[1–3] as well as in malignant processes.[4] While these investigations were still in progress Friou and Wenner[5] reported a similar elevation in rheumatic fever as determined by the mucin clot test, and Thompson and Moses[6] also found this effect in pneumonia by the clot test. Fulton, Marcus and Robinson[7] used a method based on decapsulation of a group A hemolytic streptococcus to measure the inhibition by serum and reported no significant differences between normals, and patients with rheumatoid arthritis.

The inhibiting factor which undergoes change is distinct from the specific antibody inhibitors which are elicited in response to hyaluronidase acting as an antigen. By electrophoresis at pH 8.6 it was found that the non-antibody inhibitor in the serum migrated chiefly with the albumin.[8] The antibody inhibitor would be expected to be found in the gamma globulin fraction. The factor in question appears to be a non-specific inhibitor since it is capable of inhibiting the enzyme from diverse sources, and furthermore its elevation seems to be a non-specific response to both infection and malignancy. While it is still premature to advance a theory, one might postulate that this response is a defense mechanism designed to counteract the invasiveness potentiated by hyaluronidase. While many organisms do not in themselves possess hyaluronidase, their invasiveness appears to be enhanced, nevertheless, by the presence of the enzyme,[9] and, accordingly, it is conceivable that a general response against hyaluronidase activity might be employed as a general defense mechanism.

The possibility that hyaluronidase may be involved in the invasive processes of cancer,[10–16] and the great elevation of the serum

* Aided by grants from the Division of Research Grants and Fellowships, National Institute of Health, U. S. Public Health Service, Bethesda, Md., the Medical Research Fund of the Graduate School, University of Minnesota, and the American Cancer Society, upon recommendation of the Committee on Growth of the National Research Council.

1 Glick, D., and Gollan, F., *J. Inf. Dis.*, 1948, **83**, 200.

2 Grais, M. L., and Glick, D., *J. Invest. Dermatol.*, in press.

3 Grais, M. L., and Glick, D., in preparation.

4 Hakanson, E. Y., and Glick, D., *J. Nat. Cancer Inst.*, in press.

5 Friou, G. J., and Wenner, H. A., *J. Inf. Dis.*, 1947, **80**, 185.

6 Thompson, R. T., and Moses, F. E., *Fed. Proc.*, 1948, **7**, 282.

7 Fulton, J. K., Marcus, S., and Robinson, W. D., *Proc. Soc. Am. Bact.*, 1948, **1**, 95.

8 Glick, D., and Moore, D. H., *Arch. Biochem.*, 1948, **19**, 173.

9 Duran-Reynals, F., *Bact. Rev.*, 1942 **6**, 197.

TABLE I.
Hyaluronidase Inhibitor in Blood Serum of Cancer Mice.

| Group | Strain | No. mice | Presence of milk agent | Presence of tumor | Mean inhibitor conc. (A) per cc serum | Difference of means | t* | P |
|---|---|---|---|---|---|---|---|---|
| 1 | Breeders | 13 | — | — | 19.2 | 0.8 | .20 | >.55 |
| | Ax | 11 | + | + | 18.4 | | | |
| | Aa | | | | | | | |
| 2 | Virgins | 22 | — | — | 16.1 | 0.2 | .05 | >.55 |
| | Ax | 19 | + | — | 15.9 | | | |
| | Aa | | | | | | | |
| 3 | Zb | 19 | — | — | 12.8 | 1.2 | .60 | >.55 |
| | Zz | 14 | + | + | 11.6 | | | |
| 4 | $ZbAxF_1$ | 19 | — | — | 10.1 | 3.6 | 1.45 | .15 |
| | $ZzAaF_1$ | 3 | + | + | 6.5 | | | |

* Statistical quantity used for less than 30 observations to calculate probability (P) that the differences could have resulted from chance alone.

inhibitor in metastatic carcinoma,[4] led to the present investigation of the level of the inhibitor in the blood serum of various strains of mice in relation to the genesis of mammary cancer. It was hoped that some light might be shed on the question of whether the hyaluronidase inhibitor has any significance in resistance to mammary cancer in mice.

*Materials and methods.* The mice employed were of the following strains used by Bittner and Huseby.[7]

*Aa* stock—*A* stock cancer mice nursed by mothers with *"a"* milk agent.

*Breeders*—high cancer line, with cancer when used.

Virgins—low cancer line, without cancer when used.

*Ax* stock—Decended from 1 animal of the *A* stock that had been nursed by a female of *X* or *CBA* strain. Without cancer when studied. No cancer without milk agent.

*Zz* stock—*Z* or *C3H* stock cancer mice possessing *"z"* milk agent with cancer when used.

*Zb* stock—Fostered *Z* stock mice without milk agent. Descendents from females fostered by mice without milk agent. Without cancer when used.

*Zb Ax* $F_1$—First hybrid generation of *Zb*♀ × *Ax*♂. Without cancer when used. Devoid of milk agent.

*Zz Aa* $F_1$—First hybrid generation of *Zz*♀ × *Aa*♂. High cancer incidence, both *"a"* and *"z"* milk agents present. With cancer when used.

The mice were anesthetized with ether, and 0.5–1.0 cc of blood was taken from the jugular vein of each animal. After clotting, the serum was withdrawn and stored at –25° until used for the determination of hyaluronidase-inhibitor content. The details of the viscosimetric method used, which employed hyaluronidase from bull testes and hylauronic acid from human umbilical cords, have been given in an earlier paper.[1] The value (A) expressing degree of inhibition is defined as $\left(\frac{R-Ro}{Ro}\right)$, where (Ro) equals the time in seconds for the viscosity of the reaction mixture without serum to fall to half its initial value, and (R) equals the corresponding time in seconds for the viscosity to fall to half the initial value in the presence of serum. Only 0.02 cc of serum is required in the 6 cc of total reaction mixture prepared for each measurement. The concentration of inhibitor was calculated as

10 Boyland, E., and McClean, D., *J. Path. Bact.*, 1935, **41**, 560.

11 Pirie, A., *Brit. J. Exp. Path.*, 1942, **23**, 277.

12 Hoffman, D. C., Parker, F., and Walker, T., *Am. J. Path.*, 1931, **7**, 523.

13 Duran-Reynals, F., *J. Exp. Med.*, 1931, **54**, 493.

14 Duran-Reynals, F., and Claude, A., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 67.

15 McCutcheon, M., and Cowan, D. R., *Cancer Res.*, 1947, **7**, 379.

16 Cowan, D. R., McCutcheon, M., and Zeidman, I., *Cancer Res.*, 1947, **7**, 383.

TABLE II.
Relation of Cancer Incidence in Strains of Mice to Hyaluronidase Inhibitor in Blood Serum.

| Group | Strain | No. mice | % Cancer[7] | Mean inhibitor conc. (A) per cc serum | Groups compared | Difference of means | K* | P |
|---|---|---|---|---|---|---|---|---|
| 1 | Aa breeders | 13 | 86.7 | 15.3 | 1 and 2 | 0.5 | 0.1 | >.55 |
| | | | | | 1 and 3 | 3.5 | 1.2 | .230 |
| 2 | Aa virgins | 40 | 3.9 | 14.8 | 1 and 4 | 5.8 | 2.1 | .036 |
| 3 | Zz breeders | 30 | 95.1 | 11.8 | 2 and 4 | 5.3 | 2.8 | .005 |
| 4 | ZzAa breeders | 21 | 97.6 | 9.5 | 3 and 4 | 2.3 | 1.5 | .134 |

* Statistical quantity used for 30 or more observations to calculate probability (P) that the differences could have resulted from chance alone.

(A) per cc serum.

*Results and discussion.* From the data in Table I it is apparent that within *A* and *Z* strains no significant difference was found between the inhibitor levels in the serums of mice with or without the milk agent, and with or without tumors. The differences in the incidence of cancer between the virgin *A* and *Z* strains appears to result from an "inherited hormonal factor" in the *Z* strain.[17] Bittner and Huseby[17] emphasized the possibility that the inherited susceptibility to mammary cancer may be the same in both strains, while other genes control the "hormonal factor." From the present data it would appear that there is a tendency toward an inverse relationship between the level of the hyaluronidase inhibitor in the serum and the presence of the "inherited hormonal factor". A direct relationship between the porphyrin level and the latter factor was indicated by the work of Bittner and Watson.[18]

Since the presence of milk agent or tumor had no appreciable effect on the inhibitor level in the mouse serum, the comparisons in Table II deal only with cancer incidence and the inhibitor. None of the data in Table I could be compared directly with those in Table II because different batches of enzyme and substrate were used to obtain the data for each table. Variations in the preparations do not justify direct comparisons of results obtained with different lots. A comparison of *A, Z,* and their hybrid strains reveals a tendency toward lower inhibitor levels in strains capable of higher cancer incidence as virgins and which bear the "inherited hormonal factor."

Significant differences in the inhibitor levels of Group 2 and 4, and 1 and 4 may be noted in Table II. These differences accompany, in inverse relation, the differences in cancer incidence. Within the *A* strain, large differences in the cancer incidence in breeders and virgins is not associated with a significant difference in the inhibitor level. Furthermore no important differences in the inhibitor are seen between *Aa* and *Zz* strains or the *Zz* and *ZzAa* strains.

*Summary.* 1. Within the *A, Z,* and $AZF_1$ strains of mice, no significant differences in the hyaluronidase-inhibitor levels in serum were found between individuals with and without the milk agent which determines the incidence of mammary cancer. Neither was a significant difference observed between virgins and breeders of the same strain or between individuals with and without tumors. 2. There appeared to be a tendency for the inhibitor titer to vary inversely with the strain incidence of mammary cancer in the *A, Z,* and $AZF_1$ mice. 3. A tendency toward an inverse relationship between the inhibitor level and the presence of the "inherited hormonal factor" follows.

[17] Bittner, J. J., and Huseby, R. A., *Cancer Res.*, 1946, **6**, 235.

[18] Bittner, J. J., and Watson, C. J., *Cancer Res.*, 1946, **6**, 337.

16778

# Failure of Estradiol Inactivation Products to Inhibit Pituitary Gonadotrophic Content and Secretion.

EDWIN C. JUNGCK.* (Introduced by Carl G. Heller.)

*From the Department of Physiology, University of Oregon Medical School, Portland, Ore.*

It has been postulated that the level of pituitary gonadotrophins in the blood is regulated principally by the degree of activity of the ovaries, functioning ovaries inactivating gonadotrophins, and non-functioning ovaries permitting gonadotrophins to accumulate in the circulation.[1] This hypothesis was based on observations of female rats with ovaries autotransplanted to the spleen. By this means, ovarian secretion was directed through the portal vein directly to the liver, where the estrogenic substances were inactivated sufficiently to prevent their detection in the systemic circulation. As in castrates, it was noted that vaginal cell atrophy, uterine atrophy and thymic hypertrophy occurred; however, pituitary gonadotrophic content did not rise, as in castrates, but remained at normal levels. This refuted the concept that normal levels of estrogen maintain normal pituitary gonadotrophic content, and suggested instead that pituitary gonadotrophic content is the resultant of the activity of functional ovaries upon circulating gonadotrophins.

An alternate explanation for the lack of rise of pituitary gonadotrophins is the possible influence of estrogen inactivation products upon the hypophysis. In animals with their ovaries transplanted to the spleen, degradation products of the estrogens which have passed through the liver, although lacking estrogenic activity, might conceivably depress the hypophysis.

Smith[2] found that Westerfeld's lactone, an inactivation product of estrone, increased pituitary content of gonadotrophins. Segaloff[3] has found that bisdehydrodoisynolic acid, a degradation product of estrogens, is more active when administered intrasplenically than when injected subcutaneously. Since some inactivation products of estrogens do have effects on pituitary gonadotrophic content, it seemed possible that they might be the principal controlling factor. This possibility is the subject of the present investigation.

Only estrone and estradiol have been recovered from ovarian tissue. As estrone is changed to estradiol in the process of its inactivation by the liver,[4] $\alpha$-estradiol was chosen as the estrogen for this study.

*Materials and methods.* Adult virgin female rats of the Sprague-Dawley strain weighing 200-250 g were castrated, and on the same day $\alpha$-estradiol pellets weighing 1.1 to 10.8 mg were implanted in their spleens. One group of rats received pellets subcutaneously, and another group was included as intact controls. Operated rats with pellets implanted in the spleen which spontaneously developed vascular adhesions from the spleen to the systemic circulation served as additional controls.

From 33 to 40 days after the pellets were implanted, the donor rats were killed by decapitation. Pituitary gonadotrophin content was measured by suspending each anterior pituitary gland in 6.0 cc of saline by repeatedly drawing into and expelling from a syringe, then by injecting into one 24-day-old Sprague-Dawley female rat, 1.0 cc twice daily for three days. The assay rats were killed 24 hours after the last injection. Results are tabulated in Table I.

*Discussion.* The control of pituitary gonadotrophic secretion could conceivably be

* Schering Fellow in endocrinology.

1 Jungck, E. C., Heller, C. G., and Nelson, W. O., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 148.

2 Smith, O. W., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 242.

3 Segaloff, A., *Fed. Proc.*, 1947, **6**, 399.

4 Heller, C. G., *Endocrin.*, 1940, **26**, 619.

TABLE I.

| Donor rats | | | | | | | | Recipient rats | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | α-estradiol pellets | | At autopsy* | | Uterus | | | |
| | No. of rats | Vaginal smears: Before operation | Vaginal smears: After operation | Avg wt when implanted, mg | Avg absorbed, μg/day | Appearance of uterus | Thymic wt, mg | With fluid, mg | Without fluid, mg | Ovarian wt, mg | No. of rats |
| Intact controls | 3 | cycling | cycling | — | — | normal | 183 | 185 | 111 | 20 | 3† |
| Castrate controls | 11 | ,, | atrophic | — | — | atrophic | 241 | 95 | 95 | 103 | 11† |
| α-estradiol pellet in spleen, no adhesions | 16 | ,, | ,, | 5.4 (1.1-10.2) | 21.9 (5.4-47.5) | ,, | 293 | 115 | 115 | 78 | 16 |
| Same with adhesions | 6 | ,, | estrus | 5.3 (2.3-10.8) | 19.7 (8.8-32.5) | estrus | 83 | 54 | 54 | 25 | 6 |
| α-estradiol pellet SQ | 3 | ,, | ,, | 6.2 (4.3-9.7) | 74.0 (39.4-119.0) | ,, | 38 | 34 | 34 | 16 | 3 |
| | | | | | | Uninjected recipient rats | | 36 | 36 | 13 | 63 |

* Autopsy 33-40 days after implantation of pellets.
† These values are in keeping with previous observations made on the same strain of rats.

due to estrogen inactivation products from the liver. Thus, when an animal is castrated, there are no estrogen inactivation products and the pituitary is permitted to increase markedly in gonadotrophic content. When the ovaries are present in the spleen, the pituitary gonadotrophic content would continue to be controlled by the estrogen inactivation products, thus accounting for the normal gonadotrophin levels found in these animals.

To test this hypothesis, pellets of α-estradiol were implanted into the spleens of mature female rats castrated the same day. The relationships in the experimental rats are diagrammed in Fig. 1. An α-estradiol pellet (at 1 in the figure) was implanted in the spleen. The estradiol was carried directly to the liver (at 2) and inactivated, so no active estradiol reached the general systemic circulation. (This was confirmed by the castration response elicited — hypertrophied thymus, atrophic uterus and atrophic vaginal smear.) Inactivation products of estradiol arrived in the circulation and were free to act on hypophyseal gonadotrophic content and secretion (at 3).

If the principal controlling factor of pituitary gonadotrophic content is estrogen inactivation products, then castrated female rats having pellets of α-estradiol in their spleens should have normal pituitary gonadotrophin content. If the main controlling mechanism is inactivation of gonadotrophins by the ovaries, as postulated, then the presence of estradiol pellets in the spleen should have little effect in maintaining normal pituitary gonadotrophic potency of castrated female rats.

The pituitaries of normal unoperated female rats contained sufficient gonadotrophin to stimulate recipient ovarian weights from 13 mg to 20 mg. Castrate control rat pituitaries stimulated recipient ovarian weight from 13 mg to 103 mg. The animals with α-estradiol pellets in the spleen (and no adhesions) had sufficient gonadotrophin in their pituitaries to stimulate recipient ovarian weight from 13 mg to 78 mg, which is in the castrate range. The average daily absorption of α-estradiol was 21.9 μg, several times the

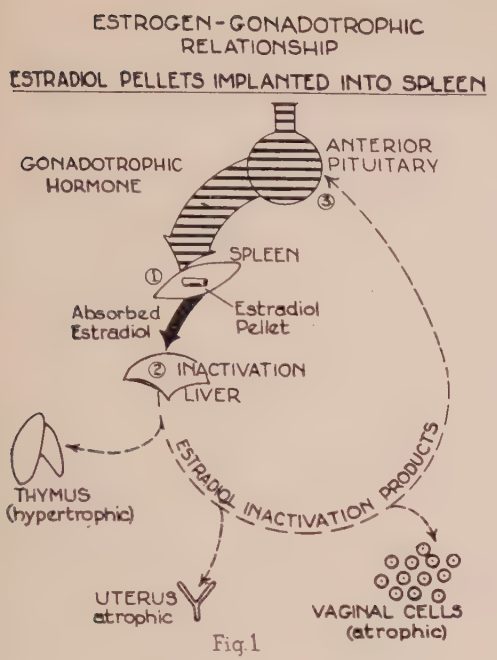


Fig. 1

dose necessary to maintain constant estrus in the intact animal. This large dose of α-estradiol was completely inactivated, as demonstrated by the atrophic uteri and vaginal smears, and the large thymus. Therefore, many times the physiological amount of inactivation products were present in the circulation. However, the pituitary gonadotrophic content rose to castrate levels.

The castrated rats with pellets implanted in the spleen which developed vascular adhesions from the spleen to the systemic circulation absorbed essentially the same amount of α-estradiol daily as the rats without adhesions (19.7 μg vs. 21.9 μg). The rats with estradiol pellets implanted subcutaneously absorbed 74.0 μg daily. In both the adhesion group and in the rats with pellets implanted subcutaneously, constant estrus was observed along with atrophy of the thymus and suppression of pituitary gonadotrophic content. These changes are noted only with unphysiologically large doses of estrogen,[1] thus indicating that larger than normal amounts of estrogen were being absorbed.

*Conclusions.* 1. The inactivation products of α-estradiol produced by passage of estradiol through the liver do not exert a significant inhibiting influence on anterior pituitary gonadotrophic secretion.

2. The absence of this effect supports the thesis that pituitary gonadotrophic content and secretion are controlled principally by ovarian inactivation of circulating gonadotrophins.

16779

## Effect of Adrenal Cortical Extract on Recovery from Severe Pneumococcic Infection in Mice.*

LOUIS TOBIAN, JR., AND ELIAS STRAUSS. (Introduced by T. R. Harrison.)

*From the Departments of Medicine and Bacteriology, Southwestern Medical College, Dallas, Texas.*

Many observations suggest that the increased adrenal cortical secretion during stress provides an increase in nonspecific resistance.[1] In patients or animals moribund from infections, it was thought that administration of large doses of adrenal cortical extract might temporarily increase resistance and thus gain time for antibacterial agents such as penicillin to effect recovery. To test this possibility two similar experiments were performed.

*Methods.* Albino Swiss mice were inoculated intraperitoneally with large numbers of Type I pneumococci. After a certain percentage of the inoculated mice had died, the survivors were placed at random into 3 dif-

* This study was supported by grants from the United States Public Health Service, and from the Lederle Laboratories Division, American Cyanamid Company.

1 Selye, H., *J. Clin. Endocrinol.*, 1946, **6**, 117.

TABLE I.
Effect of Adrenal Cortical Extract and Penicillin on Survival of Mice Infected with Pneumococci.
Treatment begun 22 hours after inoculation when 5% of mice were dead.

| Hr after pneumococcus inoculation | Group I Penicillin G + adrenal cortical extr. | | Group II Penicillin G + corn oil | | Group III Saline + corn oil | |
|---|---|---|---|---|---|---|
| | No. living | % living | No. living | % living | No. living | % living |
| 22 | 34 | 100 | 34 | 100 | 25 | 100 |
| 24 | 18 | 53 | 23 | 68 | 15 | 60 |
| 26 | 15 | 44 | 16 | 47 | 4 | 16 |
| 28 | 13 | 38 | 13 | 38 | 1 | 4 |
| 30 | 12 | 35 | 10 | 29 | 0 | 0 |
| 34 | 10 | 29 | 9 | 26 | 0 | 0 |
| 38 | 9 | 26 | 8 | 24 | 0 | 0 |
| 42 | 8 | 24 | 8 | 24 | 0 | 0 |
| 46 | 8 | 24 | 8 | 24 | 0 | 0 |
| 50 | 7 | 21 | 8 | 24 | 0 | 0 |

TABLE II.
Effect of Adrenal Cortical Extract and Penicillin on Survival of Mice Infected with Pneumococci.
Treatment begun 22 hours after inoculation when 10% of mice were dead.

| Hr after pneumococcus inoculation | Group I Penicillin G + adrenal cortical extr. | | Group II Penicillin G + corn oil | | Group III Saline + corn oil | |
|---|---|---|---|---|---|---|
| | No. living | % living | No. living | % living | No. living | % living |
| 22 | 37 | 100 | 39 | 100 | 14 | 100 |
| 24 | 30 | 81 | 35 | 90 | 14 | 100 |
| 26 | 26 | 70 | 31 | 80 | 13 | 93 |
| 28 | 22 | 59 | 30 | 77 | 11 | 79 |
| 30 | 22 | 59 | 30 | 77 | 8 | 57 |
| 34 | 21 | 57 | 29 | 74 | 3 | 21 |
| 38 | 21 | 57 | 28 | 72 | 3 | 21 |
| 42 | 20 | 54 | 28 | 72 | 0 | 0 |
| 46 | 20 | 54 | 28 | 72 | 0 | 0 |
| 50 | 19 | 51 | 25 | 64 | 0 | 0 |

ferent therapy groups.

Group I received 250 units of crystalline sodium penicillin G dissolved in 0.5 cc saline intraperitoneally every 2 hours. These mice were also injected subcutaneously with 0.2 cc of adrenal cortical extract (Upjohn's "Lipo-Adrenal Cortex") immediately after the first penicillin injection. Subsequently, at 4 and 8 hours after this injection they received an additional subcutaneous injection of 0.1 cc of adrenal cortical extract.

Group II was treated on the same dosage schedule of penicillin as Group I. However, subcutaneous injections of corn oil (Mazola) were administered instead of adrenal cortical extract.

Group III was a control group. Mice in this group received 0.5 cc of saline instead of the penicillin solution every 2 hours, as well as the same type of corn oil injections received by Group II. In both experiments the therapeutic program was carried out for 28 hours.

*First experiment.* Ninety-eight mice averaging 14 g in weight were inoculated intraperitoneally with 25,000 pneumococci per mouse. Treatment was delayed until 22 hours after inoculation when 5% of the mice were dead. The 93 survivors were divided at random into 3 groups and treated according to the plan described above.

*Second experiment.* One hundred mice averaging 20 g each were inoculated intraperitoneally with 75,000 pneumococci per

mouse. Treatment was withheld until 22 hours after inoculation when 10% of the animals had died. The 90 survivors were divided at random into 3 treatment groups as outlined above.

In addition, control experiments were performed which showed that adrenal cortical extract, corn oil, penicillin and saline injections in the dosages and schedules used in the experiments had no noticeably harmful effects on uninfected mice.

*Results and comments.* The mortality in the 3 treatment groups is indicated in Table I for the first experiment and in Table II for the second experiment. As would be expected, the groups treated with penicillin showed a much lower mortality than the controls receiving no penicillin. No further improvement in mortality was effected by additional treatment with large doses of potent adrenal cortical extract. If these experiments are applicable to clinical situations, the results do not lend encouragement to the use of adrenal cortical extracts in the treatment of patients moribund from bacterial infections.

*Summary.* Mice of varying weights were inoculated intraperitoneally with large numbers of Type I pneumococci and the infections allowed to proceed until from 5 to 10% of the animals had succumbed. The survivors were divided into groups at random and treated with penicillin and penicillin plus potent adrenal cortical extract. No improvement was obtained in the therapeutic results achieved with penicillin by the additional treatment with adrenal cortical extract.

We wish to thank Dr. E. Gifford Upjohn of the Upjohn Pharmaceutical Company for generously supplying us with Lipo-Adrenal Cortex (Upjohn).

16780

## Reproduction of Human Ulcerative Pulmonary Tuberculosis in Rabbits by Quantitative Natural Airborne Contagion.*

MAX B. LURIE AND SAMUEL ABRAMSON.†

*From the Henry Phipps Institute, University of Pennsylvania, Philadelphia.*

The purpose of this report is to outline a study in which the acquisition and development of human ulcerative pulmonary tuberculosis was closely simulated in rabbits by exposing them to the inhalation of known numbers of tubercle bacilli while breathing naturally in an experimental apparatus.

*Materials and methods.* An apparatus for airborne infection of rabbits, constructed at the Cornell University Medical School, was placed at our disposal by Dr. Eugene L. Opie. This was modified in accordance with the principles elaborated by Wells[1] and further adjusted by certain appliances directed toward improving its quantitative aspects.

Fig. 1 gives a schematic drawing of the instrument. Briefly, a fine suspension of largely isolated, virulent bovine type tubercle bacilli, freed from clumps larger than a red blood cell by filtration through Whatman number 5 filter paper, is atomized with a known volume of compressed air in unit time through a specially designed nozzle. The large droplets settle out quickly in the spraying flask. A uniform flow of droplet nuclei, containing tubercle bacilli, is delivered into a mixing device where it is diluted with room air. Thence the contaminated air is drawn through a 16-foot pipe into a chamber where the rabbits are exposed. This chamber is exhausted by the draft action of a hot flame at the bottom of a baffled chimney connected

* Aided by grants from the Commonwealth Fund and the Faculty Research Committee, University of Pennsylvania.

† Tuberculosis Control Division, Public Health Service, Federal Security Agency.

1 Wells, W. F., *Science*, 1940, **91**, 172.

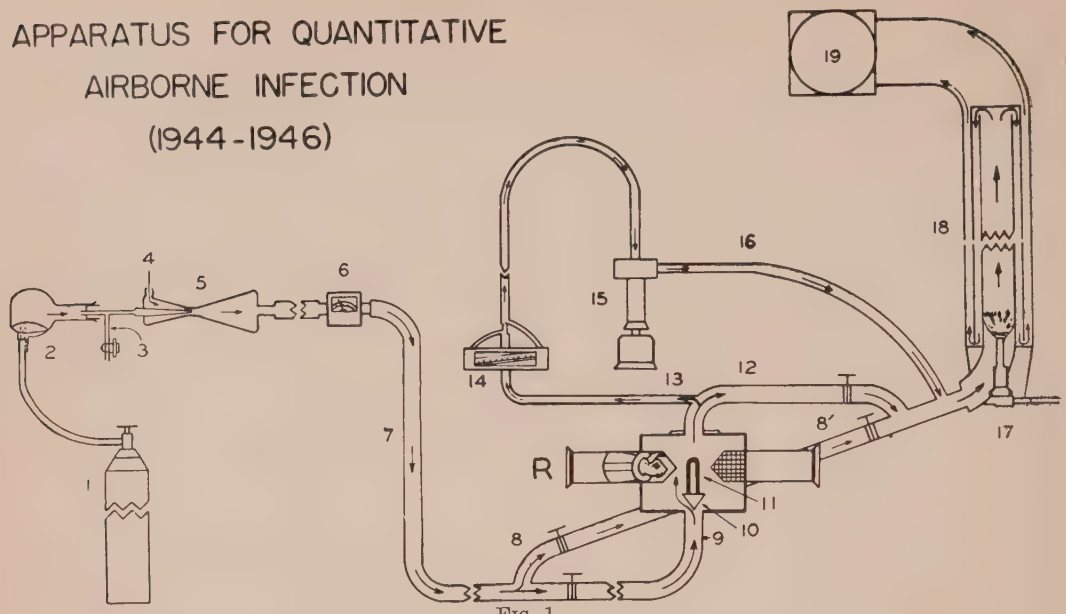



FIG. 1.

1—Compressed air tank. 2—Atomizer and spraying flask. 3—Outlet for sampling aerosol. 4—Air inflow from room. 5—Mixing device. 6—Flow-meter (liters/min.). 7—Tube which conveys infected air. 8-8′—Shunt. 9—Inlet tube to exposure chamber. 10—Spreading cone. R—Rabbit in cylinder, with head protruding into exposure chamber through rubber collar about its neck. 11—Ultraviolet lamp. 12—Outlet tube from exposure chamber. 13—Outlet for sampling exposure chamber. 14—Inclined draft gauge. 15—Wells air centrifuge. 16—Air centrifuge exhaust tube. 17—Burner. 18—Baffled incinerating chimney. 19—Exhaust fan.

to the outlet of the chamber. The incinerated air is exhausted to the outdoor atmosphere by a fan.

The concentration of tubercle bacilli in the air respired by the exposed rabbits is determined culturally[2] with the aid of a Wells air centrifuge, provided with a calibrated inclined draft gauge, which gives the exact volume of air sampled. Since the total volume of contaminated air respired by the rabbits during their period of exposure can be determined,[3] the number of bacilli to which they were exposed can be calculated.

If the bacilli in the exposure chamber are too few to be accurately determined directly, they can be estimated by ascertaining their number in the aerosol immediately after it leaves the spraying flask and before it is diluted by the inflowing room air. The dilution factor existing between the concentration of bacilli in this aerosol and that in the exposure chamber can be empirically determined by numerous preliminary calibrations. This factor depends, in part, on the volume of room air added to a unit volume of aerosol in a given time, and can be calculated from data obtained by a flowmeter which measures the rate of air flow through the system. This determination is made possible by introducing a shunt into the duct which feeds the infected air to the exposure chamber. By means of this shunt, which is provided with appropriate valves, the infected air can be made to bypass the exposure chamber in which the rabbits are placed, and in which they can breathe uninfected air while the concentration of bacilli in the aerosol is ascertained. At the desired moment, without altering the inflow of infected air and without danger to the operators, the valves can be adjusted and the contaminated air allowed to flow through the exposure chamber.

*Experimental.* Twenty-nine rabbits, of unknown genetic resistance to tuberculosis, in groups of 2 to 5, in 9 separate experiments were exposed to the inhalation of varying numbers of highly virulent bovine type tuber-

[2] Lurie, M. B., *J. Exp. Med.*, 1934, **60**, 163.

[3] Murphy, D. C., and Thorpe, E. S., *J. Clin. Invest.*, 1931, **10**, 545.

TABLE I.

Relation Between the Number of Tubercle Bacilli Respired and the Number of Tubercles Developed.

| 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| Exp. No. | No. of tubercle bacilli per l. respired air | Rabbit No. | No. of tubercle bacilli calculated as respired | No. of primary tubercles found in both lungs | Ratio between No. of tubercle bacilli respired and No. of tubercles in lungs |
| 1 | 1217 | 1 | 8,819 | 591 | 14.9 |
| | ,, | 2 | 8,041 | 973 | 8.2 |
| | ,, | 3 | 10,116 | 1,270 | 8.0 |
| 2 | 643 | 4 | 3,858 | 1,344 | 2.8 |
| | ,, | 5 | 4,629 | 833 | 5.5 |
| 3 | 468 | 6 | 2,808 | 425 | 6.6 |
| | ,, | 7 | 2,574 | 605 | 4.2 |
| | ,, | 8 | 2,808 | 397 | 7.1 |
| | ,, | 9 | 2,808 | 387 | 7.2 |
| 4 | 228 | 10 | 1,436 | 574 | 2.5 |
| | ,, | 11 | 1,276 | 397 | 3.2 |
| | ,, | 12 | 1,413 | 1,012 tubercle bacilli recovered from lungs | |
| 5 | 91 | 13 | 627 | 59 | 10.6 |
| | ,, | 14 | 664 | 82 | 8.1 |
| | ,, | 15 | 700 | 38 | 18.4 |
| | ,, | 16 | 664 | 48 | 13.8 |
| | ,, | 17 | 609 | 104 | 5.8 |
| 6 | 87 | 18 | 522 | 130 | 4.0 |
| | ,, | 19 | 609 | 59 | 10.3 |
| | ,, | 20 | 600 | 105 | 5.7 |
| 7 | 17 | 21 | 114 | 5 | 23.0 |
| | ,, | 22 | 122 | 15 | 8.1 |
| 8 | 13 | 23 | 78 | 18 | 4.3 |
| | ,, | 24 | 78 | 39 | 2.0 |
| | ,, | 25 | 74 | 16 | 4.6 |
| | ,, | 26 | 75 | 100 tubercle bacilli recovered from lungs | |
| 9 | 5 | 27 | 32 | 3 | 10.1 |
| | ,, | 28 | 32 | 1 | 32.0 |
| | ,, | 29 | 31 | 0 | — |
| | | | | Avg | 9 ± 7 |

cle bacilli of the strain Ravenel grown on a modified Lowenstein medium.[2] The number of bacillary units in the air respired by the rabbits in the individual experiments ranged from 5 to over 1200 per liter, and are listed in column 2, Table I.

Given the duration of exposure of each rabbit to the known concentration of bacilli in its respired air, the total number of bacillary units to which the rabbits were exposed can be calculated by Kleiber's formula.[4] This states that animals inhale 212 cc of air per minute per kilo of body weight to the three-quarter power in order to satisfy their oxygen needs. Column 4 lists these figures.

That the number of bacilli calculated to have been inhaled by this method is not far from the actual number of microorganisms found in the lung immediately after exposure has been noted previously.[5] It is also suggested in this table. It will be seen that in

[4] Kleiber, M., *Science*, 1944, **99**, 542.

[5] Wells, W. F., and Lurie, M. B., *Am. J. Hyg.*, 1941, **34**, Sect. B, 21.

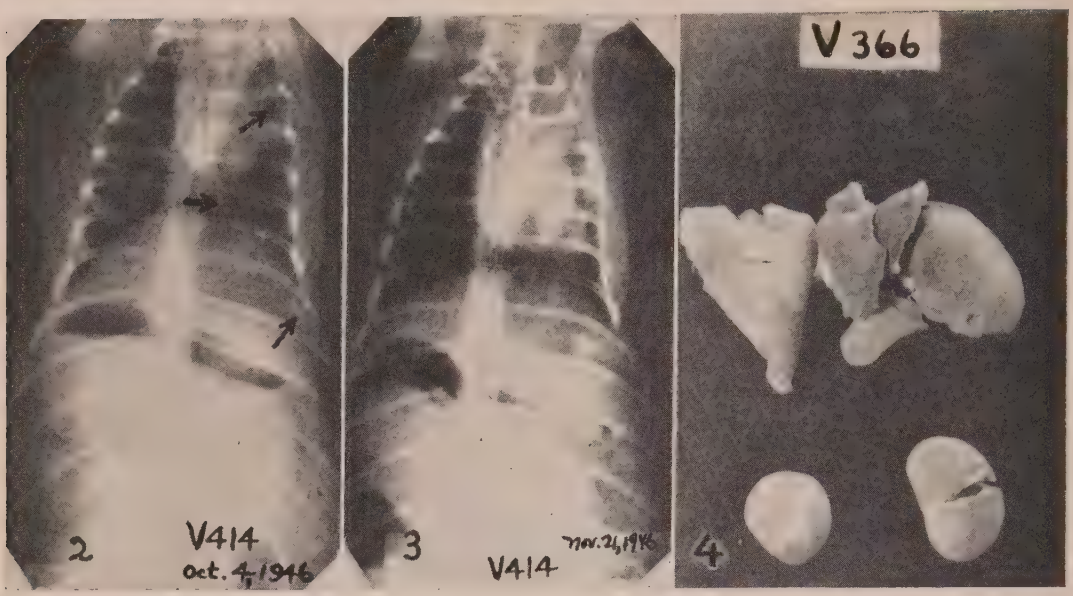



FIG. 2. Radiograph of rabbit V 414, 67 days after exposure. Three foci are visible. A walled off cavity in the third interspace and areas of consolidation in the sixth and eighth interspaces, respectively, of the left lung are indicated by arrows.

FIG. 3. Radiograph of the rabbit shown in Fig. 2, 48 days later. The progression of the disease in the left lung is evident. The right lung is normal.

FIG. 4. The lungs and kidneys of rabbit V 366 at death, 9.5 months after exposure. One of the 3 primary cavities found in the right lung is seen in the lower lobe. Infarcts of nontuberculous origin in both kidneys.

experiment 4 it was estimated that rabbit 12 had inhaled 1413 bacillary units. Actually, 1012 tubercle bacilli were recovered from its lungs immediately after exposure, as recorded in column 5. Again, in experiment 8, it was calculated that rabbit 26 had inhaled 75 bacilli. Actually, column 5 shows that 100 organisms were cultured from its lungs. Similarly close checks between the bacilli calculated as inhaled and the number recovered from the lungs have been observed since on many occasions.

Five to 6 weeks after exposure the rabbits were killed and the number of primary tubercles present in their lungs was carefully determined by excising each individual focus in both lungs. In those instances where the tubercles were too numerous for direct counting, these were estimated as follows: After removing the bronchi at their junction with the pulmonary parenchyma, both lungs were weighed. One lobe from each lung was also accurately weighed and the number of tubercles in these directly counted by enucleation of each nodule. By simple ratio the number of tubercles present in both lungs was calculated. This procedure was justified because, in these instances, the tubercles were uniformly distributed throughout both lungs.

It will be noted in columns 4 and 5 that there is a general correlation between the number of primary tubercles found in the lungs and the number of bacillary units estimated to have been inhaled. The larger the number inhaled the larger the number of primary pulmonary foci. However, there is no constant ratio between the number of bacilli required to generate a single tubercle in the different experiments, nor even in different rabbits of the same experiment inhaling the same infected air. This ratio is listed in column 6, and ranged from 2 to 32 bacillary units per tubercle in the 9 experiments. In one and the same experiment this ratio was often 3 times greater in one animal than in another. The average ratio in all the 26 rabbits was $9 \pm 7$ bacillary units per tubercle generated. Whether this ratio depends on the native resistance of rabbits or on other factors has not yet been determined.

TABLE II.

Fate of Rabbits of Race III, Sensitized with Heat-killed Tubercle Bacilli and Exposed 5.5 Months Later to the Inhalation of About 50 Virulent Bovine Tubercle Bacilli.

| Rabbit No. | Survival after exposure, mo. | Type of tuberculosis |
|---|---|---|
| V 369* | Still living, 26 | No evidence of tuberculosis. |
| U 784 | Killed, 4.7 | No tuberculosis. |
| V 366 | 9.5 | 3 small, well walled off cavities in right lung. No tuberculosis elsewhere, including hilum nodes. Large infarcts in both kidneys. |
| V 30 | 10.1 | 1 large cavity with limited bronchogenic spread in upper lobe of each lung. Hilum nodes normal. Ulcerative tuberculosis of larynx. Single miliary tubercle in one kidney. |
| V 414 | 7.3 | Unilateral ulcerative pulmonary tuberculosis with slight, contralateral lesions. Hilum nodes normal. Miliary tubercles in each kidney. Ulcerative laryngeal tuberculosis. One large tuberculous pleural nodule. Tuberculosis of one wrist joint. |
| V 267 | 6.6 | Completely excavated tuberculosis of all lobes of right lung, including the azygous lobe. Consolidation of upper lobe of left lung and bronchogenic spread to lower lobe of same lung. Hilum nodes normal. Few miliary tubercles in one kidney. |

* This rabbit was not sensitized with heat-killed tubercle bacilli before exposure.

Using the above observations as a basis, a group of 5 highly inbred rabbits of Race III, obtained from Dr. Paul B. Sawin of the Roscoe B. Jackson Memorial Laboratory, were given 6 consecutive, weekly intracutaneous injections of heat-killed bovine type tubercle bacilli, totaling 7 mg per rabbit. Five and one-half months after the last injection of heat-killed tubercle bacilli, these 5 vaccinated rabbits together with a sixth unvaccinated animal of the same race were exposed simultaneously in the apparatus for 10 minutes to droplet nuclei of virulent bovine type tubercle bacilli of the Ravenel strain derived from a culture on glycerol agar. There were 10 bacillary units per liter in the air respired by these rabbits. On the basis of the above noted Kleiber formula it was estimated that each rabbit inhaled about 5 liters of this infected air, or about 50 bacilli. Two months after exposure some of the rabbits showed radiographic evidence of sharply defined pulmonary lesions, some with cavity formation (Fig. 2); 2-3 such foci were found in these rabbits at this time. It must be noted that preliminary studies on this inbred race of rabbits had shown that they were of high genetic resistance to tuberculosis.

Table II details the results of this experiment. It will be noted that the unvaccinated rabbit, V 369, had never shown any evidence of tuberculosis and is still living 26 months after the exposure. A vaccinated animal, U 784, was killed 4.7 months after exposure and no gross tuberculosis was found anywhere in the body. The remaining 4 vaccinated rabbits showed strictly localized ulcerative pulmonary tuberculosis of varying degree of extension. In no instance were the draining tracheobronchial lymph nodes involved, and hematogenous dissemination to the rest of the body was limited to a few scarcely visible miliary tubercles in the cortex of one or both kidneys, with the single exception stated below.

The least extensive disease was seen in rabbit V 366 (Fig. 4). In its right lung there were three small, well walled off cavities, about 2-5 mm in diameter, containing tubercle bacilli. These apparently represent the original primary foci which had undergone caseation and liquefaction and had become encapsulated. There was no bronchogenic, lymphogenous, or hematogenous dissemination beyond these primary lesions when the rabbit died from renal infarction 9.5 months after infection as a result of a surgical operation.

In V 30 (Fig. 5) the disease spread by

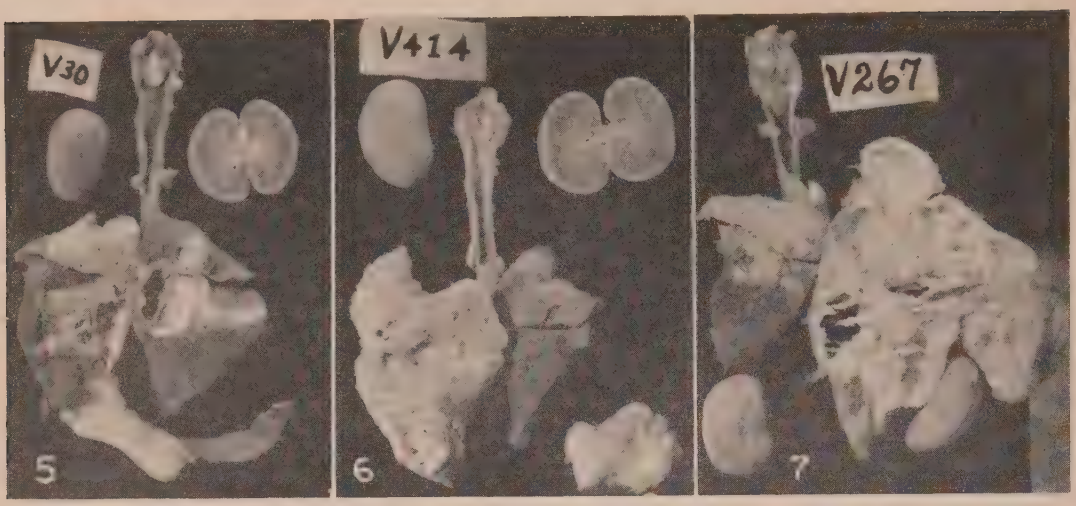



FIG. 5. The organs of rabbit V 30 at death, 10.1 months after exposure. One large cavity in each lung with limited bronchogenic spread in both. Tracheobronchial nodes and kidneys, normal. Ulcerative tuberculous laryngitis with tubular spread to the appendix, seen below left lung.

FIG. 6. Organs of rabbit V 414 at death, 7.3 months after exposure. Unilateral ulcerative tuberculosis in left lung with slight bronchogenic dissemination in the upper lobe of right lung. Tracheobronchial nodes and kidneys, normal. Ulcerative tuberculous laryngitis. Tuberculous pleural nodule in the right lower corner of photograph. The progression of the disease in this rabbit during the first 4 months of the infection is depicted in Figs. 2 and 3 above.

FIG. 7. The organs of rabbit V 267 at death, 6.6 months after exposure. Completely excavated tuberculosis of all lobes of right lung, including azygous lobe which is depicted just above the right kidney. Consolidation of upper lobe of left lung and bronchogenic spread to lower lobe of the same lung. Tracheobronchial nodes, normal. The few, minute, miliary tubercles in the kidneys cannot be seen.

bronchogenic dissemination to a considerable portion of both lungs from a single large cavity in each. The rabbit was asphyxiated by an ulcerative tuberculous laryngitis 10.1 months after infection. In the next rabbit of this series, V 414, the disease extended to a greater degree. Fig. 2 and 3 show the progression of the disease in the lungs during the first 4 months of the infection. Fig. 6 illustrates the character of the tuberculosis at death. There was a unilateral destructive phthisis with slight contralateral lesions. This rabbit also died with tuberculous, ulcerative laryngitis, 7.3 months after its infection. There was some pleural tuberculosis and one wrist joint was involved. Finally, the disease extended most rapidly in rabbit V 267, which died 6.6 months after exposure to the inhalation of 50 tubercle bacilli from complete excavation of all the lobes of the right lung including the azygous lobe. Fig. 7 illustrates the resulting thick walled cavities, the caseous consolidation of the upper lobe of the opposite lung and the bronchogenic spread to the lower lobe of the same lung.

Thus, a group of 6 rabbits of the same highly inbred, genetically resistant family showed all the degrees of resistance as seen in man; from failure of the disease to take root at all, to the formation of primary lesions which did not extend beyond their site of inception, to limited bronchogenic dissemination from the primary ulcerative foci, to unilateral ulcerative phthisis, and finally, to a rapidly progressive ulcerative tuberculosis which had destroyed one lung and was progressing from the upper to the lower lobes in the contralateral lung. The similarity of these observations to the varying types of ulcerative pulmonary phthisis as seen in man is striking.

While these animals all showed a localization of the disease which is characteristic of resistant rabbits, as evidenced by their capacity to limit the infection to the portal of entry, the lung,[6] they varied markedly in

their ability to restrict the dissemination of the disease by bronchogenic spread.

It is noteworthy that there was evidence in some of the rabbits that 50 inhaled bacilli had given rise to about 3 primary pulmonary lesions. These 3 pulmonary lesions were seen in rabbit V 366 at death and, in rabbit V 414 (Fig. 2), 3 foci were identified in the radiograph of its lungs 2 months after infection. It is noted above that an average of $9 \pm 7$ tubercle bacilli were necessary to generate a single tubercle in the preliminary experiments. Therefore, taking the larger figure, 16, as applicable in this series, 3 primary pulmonary foci would be expected from these 50 inhaled bacilli. The larger ratio is likely for this group since these rabbits, unlike the former animals, were slightly immunized with heat-killed tubercle bacilli prior to exposure.

*Summary.* A modification of an apparatus for quantitative airborne infection as devised by Wells is described. Its use has indicated that, while the number of tubercles generated in the lung is to a certain degree proportional to the number of droplet nuclei of tubercle bacilli inhaled, there is no constant ratio between the number of tubercle bacilli arrested in the lung and the number of primary tubercles generated therein, even when the rabbits breathe the same infected air simultaneously.

By exposing vaccinated highly inbred, genetically resistant rabbits to the inhalation of known small numbers of virulent bovine type tubercle bacilli it was possible to reproduce various types and phases of human localized ulcerative pulmonary tuberculosis of the adult or reinfection type. These phases ranged from complete resistance to the infection to varying degrees of bronchogenic dissemination from excavated foci to one or both lungs. There was little or no lymphogenous or hematogenous dissemination from the pulmonary portal of entry in these rabbits.

It is a pleasure to acknowledge the aid given us by Mr. R. J. Ott of the Philadelphia Gas Co., in calibrating the inclined draft gauge, and that of Mr. Peter Zappasodi for the photographs in this paper.

[6] Lurie, M. B., *Am. Rev. Tuberc.*, 1941, **44**, Suppl. 1.

16781

## Failure of Antihistaminic Drugs to Reduce Reactive Hyperemia in Man.*

MILTON LANDOWNE† AND WALTER S. THOMPSON, JR.

*From the Department of Medicine, University of Chicago.*

The physiological response of regional dilatation consequent to circulatory arrest has been designated as reactive hyperemia. The studies of Lewis and Grant[1] and Goldblatt[2] presented evidence indicative that this is a local response and the former suggested that the accumulation of a chemical was responsible for the vasodilatation. Later Lewis[3] labeled this agent H-substance and defined it as "any substance (or substances) that is liberated by the tissue cells and exerts on the minute vessels and nerve endings an influence culminating in the triple response". Subsequently others[4–6] reported finding increased amounts

* Aided by a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

† Present address: Cardiovascular Research Unit, Veterans Administration Hospital, Washington. D.C.

[1] Lewis, T., and Grant, R., *Heart*, 1925-26, **12**, 73.

[2] Goldblatt, H., *Heart*, 1925-26, **12**, 281.

[3] Lewis, T., The Blood Vessels of the Human Skin and Their Responses, 1927, London, Shaw and Sons.

[4] Barsoum, G. S., and Gaddum, J. H., *J. Physiol.*, 1935, **85**, 13P.

of histamine, or of a substance with the "biological properties of histamine", in the venous blood during reactive hyperemia and concluded that this was responsible, at least in part, for the vasodilatation. Emmelin *et al.*[7] and Kwiatkowski,[8] using the same or modifications of the same method of analysis for histamine, were unable to confirm these results. They stated, however, that their findings did not exclude the possibility that histamine is formed *in situ* and plays a role in the development of reactive hyperemia, as the possibility remains that it might not readily diffuse away from the site of action.

Despite certain objections,[9] Lewis' studies and the subsequent work have led to the generally accepted hypothesis that the vascular response in reactive hyperemia is due primarily to the accumulation of histamine or a histamine-like substance. The recent development of the antihistaminic drugs and the establishment of their histamine antagonizing property suggested a method whereby the validity of this concept might be tested in man. While this work was in progress a report of a similarly directed study in the cat appeared.[10]

*Method.* Six subjects were studied. These included 5 male patients hospitalized for reasons other than cardiovascular disease and one hypertensive female patient before and 24 days after combined nephrectomy and unilateral sympathectomy. Measurement of blood flow in the foot and distal portion of the leg was made by means of a venous occlusion volume recording plethysmograph according to principles previously described.[11] This entailed placing the part of the limb to be tested within an airtight chamber. At the proximal margin of the plethysmograph a cuff 4 cm wide was placed around the leg and used to produce venous occlusion. A cuff 17 cm wide was placed about the thigh for the purpose of producing arterial occlusion. Both cuffs were attached to tanks in such a manner that by means of a system of valves any desired pressure could be rapidly obtained or released. The majority of studies were made after the subject had an average hospital breakfast, and they extended over 2 to 3 hours. Temperatures within the plethysmographic chamber were between 25.6° and 29.4° C. After at least 30 minutes of rest in the supine position determinations of resting flow were made as frequently as every 20 seconds. Reactive hyperemia was produced by the inflation and release of the arterial occlusion cuff. The cuff was rapidly inflated to approximately 220 mm Hg and the occlusion was maintained for periods of one-half to 10 minutes. Just prior to release the venous occlusion cuff was inflated and thus the initial inflow rate immediately following the release of arterial occlusion could be recorded with minimal distortion. The initial inflow rate is taken as representative of the degree of vasodilatation resulting from the occlusion. The subsidence of the vasodilatation was noted by repeating determinations of flow as often as every 15 seconds. At least one minute after subsidence of hyperemia the arterial occlusion was repeated, until a number of measurements of hyperemia were made. After completion of these control observations under normal conditions, "benadryl" (beta-dimethyl-aminoethyl-benzhydryl ether hydrochloride) 10 to 50 mg intravenously, or "pyribenzamine" (beta-dimethyl-aminoethyl - 2 - pyridyl-benzyl ammonium chloride) 50 mg orally, was administered. Observations were repeated at intervals for more than an hour.

The volume of the portion of the limb tested was measured by water displacement, and flows were expressed in terms of cc/min./100 cc limb. In 2 of the subjects intradermal injections of 0.1 cc containing 0.01 to 0.1 $\gamma$ of histamine base were used to demonstrate

[5] Barsoum, G. S., and Smirk, F. H., *Clinical Science*, 1936, **2**, 353.

[6] Anrep, G. V., Barsoum, G. S., Salama, S., and Souidan, Z., *J. Physiol.*, 1944, **103**, 297.

[7] Emmelin, N., Kahlson, G., and Wicksell, F., *Acta Physiol. Scand.*, 1941, **2**, 110.

[8] Kwiatkowski, H., *J. Physiol.*, 1941, **100**, 147.

[9] Hamilton, W. F., Chapter 38 in Howell's Textbook of Physiology, edited by J. F. Fulton. 15th Ed., 1946, Philadelphia, W. B. Saunders.

[10] Emmelin, K., and Emmelin, N., *Acta Physiol. Scand.*, 1947, **14**, 16.

[11] Landowne, M., and Katz, L. N., *Am. Heart J.*, 1942, **23**, 644.

TABLE I.

Effect of Antihistaminic Drug Upon Blood Flow in the Foot Leg at Rest and During Reactive Hyperemia.

| | Blood flow cc/min./100 cc limb (Each value is the average of the number of measurements indicated in parentheses) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Initial inflow after release of occlusions maintained for | | | | | | |
| Subject and drug* | Resting flow | 30 sec. | 1 min. | 2 min. | 3 min. | 4 min. | 5 min. | 10 min. |
| Pr. before | 3.60 (20) | 5.7 (3) | 6.8 (3) | 7.8 (1) | 8.7 (2) | | | |
| after 50 mg B | 2.41 (22) | 5.5 (2) | 7.7 (1) | 8.5 (1) | 9.4 (1) | | | |
| Hu. before (right) | 0.78 (21) | | | | | 12.0† (1) | 13.1 (2) | |
| after 50 mg P | 1.33 (22) | | | | | | 12.9 (2) | |
| Hu. before (left)‡ | 6.56 (14) | 15.0 (1) | 12.4 (2) | | 12.8 (1) | | | |
| after 20 mg B | 6.04 (7) | 12.8 (2) | 14.5 (3) | | 13.8 (1) | | | |
| Be. before | 4.43 (8) | | | | | | 8.6 (1)† | 10.2 (1) |
| after 20 mg B | 3.53 (9) | | | | | | | 10.5 (3) |
| Bo. before | 0.76 (28) | | 3.1 (2) | 5.3 (2) | 6.3 (2) | | | |
| after 20 mg B | 0.75 (19) | | 5.3 (2) | 5.7 (1) | 7.9 (1) | | | |
| Jo. before | 1.35 (30) | 2.0 (2) | 4.0 (2) | 5.4 (2) | 5.5 (2) | | | |
| after 30 mg B | 1.21 (9) | 2.3 (4) | 5.3 (2) | 6.1 (2) | 6.0 (2) | | | |
| Cl. before | 1.45 (13) | 2.4 (2) | 3.2 (1) | 6.3† (2) | 8.4 (2) | | | |
| after 30 mg B | 3.10 (11) | 3.3 (2) | 4.9 (3) | | 8.0 (1) | | | |
| Average before | 2.70 | 6.29 | 5.91 | 6.14 | 8.31 | | 13.1 | 10.2 |
| after | 2.62 | 5.98 | 7.54 | 6.77 | 9.00 | | 12.9 | 10.5 |

* B = intravenous benadryl; P = oral pyribenzamine.

† Not included in average.

‡ Sympathectomized.

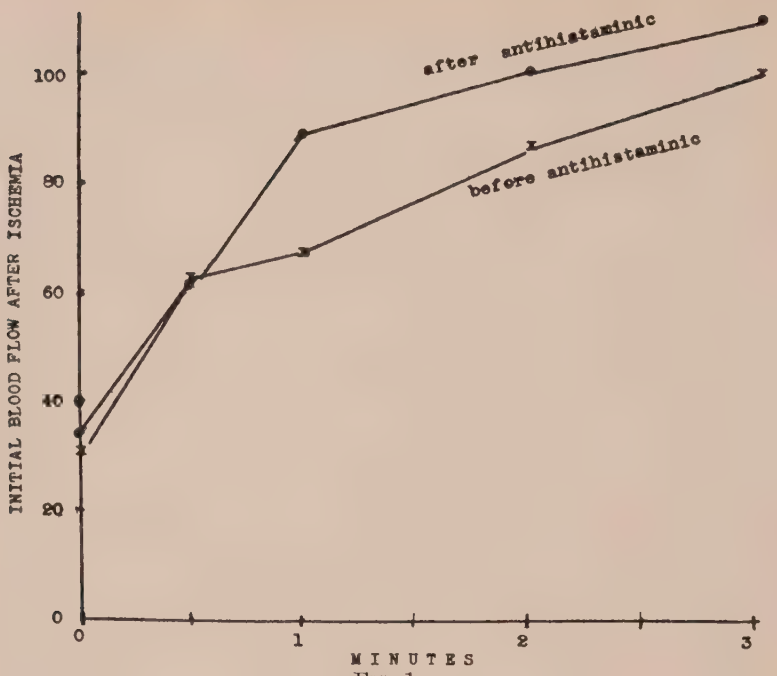



FIG. 1.

The effect of an antihistaminic drug upon blood flow at rest and during reactive hyperemia following arterial occlusion of ½, 1, 2, and 3 minutes, respectively. Averaged data from five subjects. The "control" hyperemia after three minutes of ischemia has been arbitrarily given the value of 100.

the effectiveness of the antihistamine drug. This was done simultaneously with the blood flow studies.

*Results.* Average blood flows recorded before and after the administration of the antihistaminic drug during the resting state and at the onset of reactive hyperemia are summarized in Table I. Each value for resting flow represents the average of seven to 30 determinations, a total of 134 before and 99 after the drug. The average difference in average resting flows before and after administration of the drug is 0.08 cc, a decrease of 3%. The average of the per cent difference in each subject is plus 16%, due to low resting control flows in two cases.

Each value for hyperemia represents the flow after either one to 4 periods of occlusion of the duration indicated. Hyperemia was measured 39 times before and 36 times after the drug. Nineteen grouped comparisons were made. In only 4 of these was a decrease in flow noted after administration of the antihistaminic drug. In all other comparisons greater hyperemic flow was recorded after giving the drug than before. The average change in hyperemia for all groups was 0.65 cc/min. (range ± 2.20 cc/min.). The average change for each subject was 0.54 cc/min. (range −0.20 to + 1.43 cc/min.); and the average change after occlusions of like duration was 0.46 cc/ min., or 5.5%. The average percentage change in each of these categories of comparison was between 10 and 15%.

In Fig. 1 are presented the averaged results in 5 experiments before and after the administration of the antihistaminic drug comparing flow at rest and during reactive hyperemia where arterial occlusions of one-half, one, 2 and 3 minutes respectively were used. The initial hyperemic flow after 3 minutes of occlusion before the drug was given is arbitrarily taken as a comparison standard of 100.

The duration of reactive hyperemia was brief, particularly after the shorter occlusions. Because of the normal fluctuations in resting

TABLE II.
Effect of Antihistaminic Drug Upon Whealing Produced by Intradermal Histamine.
(0.1 cc of a concentration of 1.0 γ per cc of histamine base was injected intradermally before and 17 min. after 30 mg of "Benadryl" intravenously.)

| Min. after injection | | Before drug | After drug |
|---|---|---|---|
| 3 | size of wheal, mm | 25 × 14 | 15 × 12 |
| | " " erythema, mm | 30 × 35 | 20 × 20 |
| 13 | wheal | 22 × 20 | 15 × 14 |
| | erythema | 40 × 45 | 33 × 33 |
| 23 | wheal | 22 × 20 | 15 × 12 |
| | erythema | 47 × 55 intense | 33 × 33 fading |

flow, quantitation of either the total amount or of the duration of the increased circulation is not feasible under these conditions. No qualitative differences were observed in these aspects of reactive hyperemia after antihistaminic drug.

In comparing the period before administration of the antihistaminic drug with that afterward it will be noted that no significant effect of the drug upon blood flow is demonstrated. In some experiments there was a slight average increase in resting flow, and a comparable average increase in hyperemic flow. The air temperature within the plethysmographic chamber rose 0.5° to 2.5°C, an average increase of 1.1°C. The increase in local temperature, further relaxation of the subject, and any effects of specific dynamic action are factors which may account for a slight increase in flow during the latter part of an experiment.

The effectiveness of the antihistaminic agents used is indicated by the illustrative experience presented in Table II. The wheals and surrounding erythematous halo were smaller in size and persisted for a shorter period of time when histamine in graded dosage was injected after the administration of the drug.

*Discussion.* The data presented demonstrates the failure of antihistaminic drugs to reduce the vasodilatation of reactive hyperemia in the human limb. This confirms the work of Emmelin and Emmelin[10] who were unable to show in the cat that antihistaminic drugs reduced the hyperemic volume increase following circulatory arrest.

In our studies an antihistaminic drug was carried to the tissues by means of the circulation. The concentration attained was sufficient to decrease the *local* intradermal whealing effect of 0.1 cc of concentration of 1.0 γ per cc of histamine base. Lewis considered[3] that histamine introduced into the skin acted upon the walls of the minute vessels. Emmelin and Emmelin[10] showed that the effect of intra-arterial histamine upon the minute vessels of the cat's hind limb could be reduced by an antihistaminic drug. Thus *exogenous* histamine, applied either from the outside or from within the lumen of the vessel, may have its effect upon the vessel wall reduced by the antihistaminic drug. The mechanism of the histamine blocking ability of these drugs is not known, but it has been suggested[12] that it is due to a competitive or combining property of the drug at the site of action of histamine rather than with histamine itself. This site of action would be at some portion of the cell surface or within the particular cells concerned in effecting the response. The inhibition of the action of *exogenous* histamine by an antihistaminic drug would indicate that the antihistaminic had reached this site of action. In this event, the effect of *endogenous* histamine, if produced outside or within the particular cells of the blood vessels which cause the vasodilatation of reactive hyperemia, should also be inhibited by circulating antihistaminic drugs.

The alternative possibility, that the effector

12 Friedlaender, S., and Feinberg, S. M., *J. Allergy*, 1946, **17**, 129.

cell is not penetrated by the antihistaminic drug, requires that the site of action of histamine be at a surface to which *exogenous* histamine is carried, by the circulation, by penetration through cells, or by passage between cells. In this circumstance it is conceivable that *endogenous* histamine may, by arising within the cell, reach and act upon the surface in a manner not open to inhibition by the agent used. This is unlikely. "Benadryl" is a small, ionizable molecule which readily passes from the circulating blood to the extracellular spaces. There is no evidence to indicate that it is unable to traverse the cellular structure of the vessel wall in its passage. Clinical studies[13] have shown that those allergic manifestations held to be due to an increase in *endogenous* histamine activity are reduced by antihistaminic drugs. This requires that the drug reach the site of action of the *endogenous* histamine liberated in these disorders.

The term "antihistaminic" indicates that these drugs possess the ability to inhibit the biological effects of histamine. It would be expected that a "histamine-like" substance "with the biological properties of histamine" would be similarly inhibited. This cannot be determined, of course, until such substance is actually demonstrated.

Thus the failure of antihistaminic drugs to reduce the vasodilatation of reactive hyperemia in man can be considered to mitigate strongly against the generally accepted concept that histamine or a histamine-like substance is the primary factor in the vasodilatation of reactive hyperemia.

The unmodified persistence of the capacity for reactive hyperemia after sympathetic denervation indicates, as Lewis pointed out,[3] that this phenomenon is not dependent upon sympathetic integrity. The antihistaminic drug failed to modify reactive hyperemia in the sympathectomized limb. This demonstrates that the drug does not normally tend to evoke a compensatory reduction in sympathetic activity which could mask an effect on reactive hyperemia.

*Summary and Conclusions.* 1. In 6 subjects reactive hyperemia was produced in the legs by release of arterial occlusion maintained for from one-half to 10 minutes. Blood flow in the foot and distal portion of the leg was measured with a venous occlusion plethysmograph. The initial inflow rate immediately following release of arterial occlusion is taken as representative of the degree of vasodilatation resulting from the occlusion.

2. After administration of effective amounts of antihistaminic drugs the reactive hyperemia was not diminished.

3. The mechanism of reactive hyperemia has not been shown to be due to release of histamine or "H-substance." It is as yet unexplained.

[13] Feinberg, S. M., *J. A. M. A.*, 1946, **132**, 702.

16782 P

## Nucleic Acid Content of Chromosomes of Normal and Leukemic Mouse Spleen.*

MARY L. PETERMANN AND ETHEL J. MASON. (Introduced by C. P. Rhoads.)
*From the Sloan-Kettering Institute for Cancer Research, New York City.*

It has recently been shown by Mirsky and Ris[1] that chromosomes may be isolated from the resting nucleus. These isolated chromosomes are made up chiefly of desoxypentose nucleic acid (DNA), histone, and the

* The expenses of this investigation were defrayed by grants from the Office of Naval Research, the National Cancer Institute of the United States Public Health Service, the Finney-Howell Foundation, and the James Foundation of New York, Inc.

[1] Mirsky, A. E., and Ris, H., *J. Gen. Physiol.*, 1947, **31**, 1, 7.

TABLE I.
The Nucleic Acid and Nitrogen Content of the Whole (W) and Residual (R) Chromosome Fractions of Normal and Leukemic Mouse Spleen.

| | Exp. No. | | Nitrogen—R/W, % | DNA, $\gamma/\gamma$ N | PNA, $\gamma/\gamma$ N |
|---|---|---|---|---|---|
| Normal | 1 | W | | 2.45 | |
| | | R | 14 | 0.15 | 0.18 |
| | 2 | R | — | 0.13 | 0.19 |
| | 3 | W | | 2.08 | |
| | | R | 18 | 0.05 | 0.19 |
| Leukemic | 1 | R | 14 | 0.17 | 0.32 |
| | 3 | W | | 2.77 | |
| | | R | 23 | 1.21 | 0.36 |
| | 4 | W | | 2.52 | |
| | | R | 15 | 0.19 | 0.45 |

"residual chromosomes," which contain protein, pentose nucleic acid (PNA), and possibly a small amount of DNA. In this laboratory the whole and residual chromosomes of normal and leukemic mouse spleen have been isolated by the method of Mirsky and Ris.[1] The preparations have been examined microscopically and analyzed chemically for their DNA, PNA, and nitrogen contents.

*Materials and methods.* Mice of the Akm strain, about 3 months of age, were used. When injected with leukemic spleen minced in saline (strain 9421) they developed advanced leukemia in 8 or 9 days. The mice were sacrificed by spinal fracture, and the spleens removed immediately. The spleens of the normal controls averaged 180 mg in weight, while the leukemic spleens averaged 600 mg in weight. About 9 g of spleen were homogenized in 0.88 M sucrose, for cell fractionation studies which will be described elsewhere.[2] The nuclear fraction was resuspended in 0.15 *M* NaCl. The nuclei were broken in a refrigerated Waring mixer and the chromosomes isolated.[1] The progress of the isolation was followed microscopically by smears stained with Wright's stain.

Samples of the whole chromosomes were fixed and stained with Feulgen-light green and by other methods, and examined microscopically for traces of cytoplasm.† The nucleohistone was removed by extraction with *M* NaCl until the washings showed practically no absorption of light at 260 mμ. The residual chromosomes were suspended in *M* NaCl. Nitrogen was determined by Nesslerization.[3] The nucleic acids were extracted with hot trichloroacetic acid and analyzed for DNA by the diphenylamine test and for PNA by the orcinol test.[4]

*Results.* The whole chromosome fraction was fairly free of light green-staining material on microscopic examination. The residual chromosomes were badly clumped and could not be examined in detail.

Chemical analysis (Table I) showed that the DNA content of the leukemic chromosomes was the same as or slightly higher than that of the normal chromosomes. Both sets of values correspond to a DNA content of about 37% of the total chromosome, which is similar to the values found for "chromatin threads" from leukemic mouse spleen and rat leukemic tumors[5] and for whole chromosomes from calf thymus.[1]

The PNA content of the whole chromosome fraction, as determined by the orcinol reaction in the presence of a large excess of DNA, is only an approximate value. Only traces were found in the normal chromosome fraction, and slightly more (0.1 $\gamma$ per $\gamma$ of N) in that from leukemic spleen.

The residual chromosome fraction from

2 Petermann, M. L., Alfin-Slater, R. B., and Larack, A. M., *Cancer*, in press.

† Through the courtesy of Dr. John J. Biesele.

3 Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric technics and related methods for the study of tissue metabolism, Minneapolis, 1945.

4 Schneider, W. C., *J. Biol. Chem.*, 1945, **161**, 293; 1946, **164**, 747.

5 Claude, A., and Potter, J. S., *J. Exp. Med.*, 1943, **77**, 345.

both normal and leukemic spleens contained about 17% of the nitrogen of the whole chromosome fraction (Table I). The PNA contents, on the other hand, were strikingly different. Whereas the residual chromosome fraction from normal mouse spleen contained only 0.18-0.19 $\gamma$ of PNA per $\gamma$ of nitrogen, that from leukemic spleen contained 0.32 to 0.45 $\gamma$ of PNA per $\gamma$ of nitrogen, an increase of nearly 100%.

*Discussion.* It is of great importance to determine whether the excess PNA found in the residual chromosome fraction of leukemic spleen is an integral part of the chromosome structure or is contained in some other constituent of the preparation. The cytoplasm of the leukemic spleen cells contained large amounts of PNA.[2] That any significant amount of cytoplasm was left in these preparations seems unlikely, however, since careful microscopic observation failed to reveal it. The whole "nuclear fraction" of the leukemic spleens, however, was also extremely rich in PNA.[2] This is in agreement with the findings of Thorell,[6] who has shown, by ultraviolet spectrophotography, that the nucleus of the leukemic white cell contains 2 to 4 nucleoli rich in PNA, which are absent from the normal mature white cell. Since nucleoli sometimes remain attached to isolated chromosomes,[1] it is probable that some of the excess PNA found in the leukemic residual chromosome preparations is located in associated nucleoli; how much cannot be decided until preparations are obtained which do not clump so badly, and can be examined microscopically.

*Summary.* The residual chromosome fraction isolated from leukemic mouse spleen contained from 0.32 to 0.45 $\gamma$ of pentose nucleic acid per $\gamma$ of nitrogen, while that from normal spleen contained 0.18-0.19 $\gamma$ per $\gamma$ of nitrogen.

[6] Thorell, B., Studies on the formation of cellular substances during blood cell production. London, 1947.

16783

## Isolation of a Streptomycin-Resistant Organism Capable of Utilizing Streptomycin for Growth.

GLADYS L. HOBBY AND NANCY DOUGHERTY.

*From the Biological Laboratory of Chas. Pfizer & Co., Inc., Brooklyn, N. Y.*

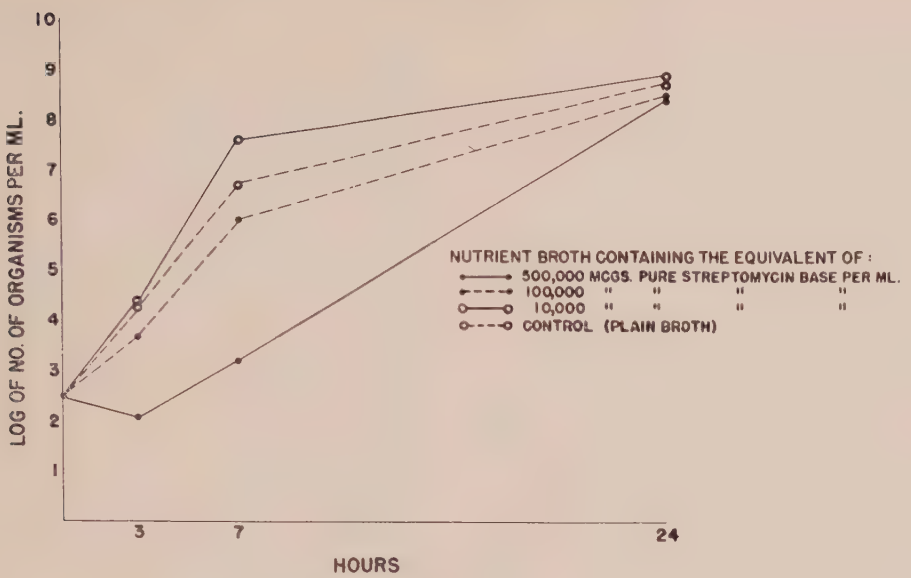
Since the introduction of streptomycin in 1944, the appearance of streptomycin-resistant variants in otherwise sensitive strains of microorganisms has been observed repeatedly. Miller and Bohnhoff[1] reported the isolation of variants of meningococci not only resistant to streptomycin but capable of growing only in its presence. These organisms were dependent upon streptomycin for multiplication both *in vitro* and *in vivo*. Paine and Finland,[2] described dependent variants of other bacterial species including *Staphylococcus aureus, E. coli, Ps. aeruginosa,* and *P. morgani.* More recently, 13 streptomycin-dependent strains of *M. tuberculosis* have been isolated in our own laboratory[3] from mice infected with the $H_{37}Rv$ strain and subsequently treated with streptomycin for a period of 31 days. The possibility that these organisms are capable of utilizing the nitrogen of streptomycin for growth has been suggested.

In the present communication, the isolation of a streptomycin-resistant organism capable of growing both in streptomycin as the sole source of nutrient and in broth containing no streptomycin is discussed.

[1] Miller, C. P., and Bohnhoff, M., *Science,* 1947, **105**, 620.

[2] Paine, T. F., and Finland, M., *Science,* 1948, **107**, 143.

[3] Lenert, T. F., and Hobby, G. L., *Am. Rev. Tuberculosis,* 1949, in press.




GRAPH 1.

Effect of streptomycin on the growth of a streptomycin-resistant gram negative rod (strain SMR).

*Isolation.* An aqueous solution of a highly purified form of streptomycin sulfate, prepared through an intermediate crystalline stage, in a concentration equivalent to 375,000 $\mu$g of pure streptomycin base per ml, was allowed to stand at room temperature for a period of several days. Subcultures were subsequently made from the solution (1) by streaking 0.05 ml of the undiluted solution onto beef infusion agar and onto Sabaraud's agar, and (2) by inoculating 0.1 ml of the undiluted solution and a $10^{-8}$ dilution of the solution into beef infusion broth and into fluid thioglycollate medium. Incubation was carried out at 5°C, 22°C, and 37°C. Luxuriant growth appeared in all cultures incubated at 22°C and at 37°C. No growth occurred at 5°C.

*Cultural characteristics.* Morphologically the organism is a short plump gram negative rod with rounded ends and varying slightly in size. At times the organism may appear almost coccoid in form. It is nonmotile and produces no spores.

The organism grows readily in diffuse form under aerobic or facultative anaerobic conditions in liquid media.* Growth occurs at pH ranging from 6.0 to 7.8 and at room temperature (22°C) and 37°C; no growth occurs at 5°C.

On plain or blood agar the organism grows in the form of a smooth opaque grayish-white colony, approximately 3-4 mm in size. It is nonhemolytic. On glucose or Sabaraud's agar, it grows in the form of a confluent viscous or mucoid slime.

Growth occurs rapidly in the presence of glucose, maltose, sucrose, levulose, arabinose, and d-galactose with the formation of acid and gas. In the presence of xylose, acid without gas is formed, while in the presence of lactose (0.1%), growth is slow and no acid is produced.

Growth occurs readily on sodium citrate agar. The organism is capable of reducing nitrates to nitrites. Acetyl methyl carbinol is not formed. In Voges-Proskauer-Methyl-Red broth, a powerful reducing substance is produced. In distilled water† and in distilled

* It is of interest to note that this organism is incapable of producing detectable amounts of penicillinase. It is sensitive to 80 units of penicillin per ml and to the equivalent of 40 $\mu$g of polymyxin B hydrochloride per ml.

† Baxter sterile distilled water was used in all experiments throughout this study. Analyses indicated a content of less than 10 p.p.m. total solids.

water† containing 3% methyl or ethyl alcohol, growth occurs rapidly within 24 hours.

Although morphologically suggestive of the Pseudomonas group of organisms, the data above are not sufficient to place this organism within this species.

*Streptomycin Resistance.* In view of the fact that this organism was originally isolated from a solution containing a high concentration of streptomycin, it is apparent that it is highly resistant to this antibacterial agent.

Graph I shows the rate of growth of the organism in broth containing varying concentrations of highly purified streptomycin. Highly purified streptomycin sulfate was diluted to 10,000, 100,000, and 200,000 $\mu$g of pure streptomycin base per ml. Plain broth was used as control. An 18-hour broth culture of the streptomycin-resistant organism was diluted in broth to a dilution of $10^{-5}$, and 0.5 ml of this was inoculated into tubes containing 4.0 ml of each of the above solutions of streptomycin in broth. Incubation was carried out at 37°C. The number of organisms per cc was determined by colony counts at 0, 3, 7, 24 hours. No inhibition of growth was observed in the presence of streptomycin in concentrations equivalent to 10,000 or 100,000 $\mu$g of pure base. A concentration of streptomycin equivalent to 500,000 units of pure base produced a temporary lag in the growth of the organism, lasting for a period of 3 to 7 hours only. After 24 hours incubation, the number of organisms per ml was as great in the presence of the equivalent of 500,000 $\mu$g of pure streptomycin base per ml as in broth containing no streptomycin.

*Utilization of streptomycin for growth.* The fact that this organism was originally isolated from a solution of streptomycin in water suggested that the organism must be capable of utilizing streptomycin for growth.

Highly purified streptomycin sulfate was diluted in sterile distilled water to a concentration equivalent to 333,000 $\mu$g of pure streptomycin base per cc. Four cc of this solution was pipetted into each of 3 tubes. As control, 4 ml of sterile distilled water was pipetted into each of 3 additional tubes. An 18-hour broth culture of the streptomycin-resistant strain was centrifuged and the sedimented organisms washed twice with sterile distilled water to remove traces of broth. The organisms were then resuspended in a volume of sterile distilled water equal to that of the original broth culture. The aqueous suspension of organisms was then diluted to $10^{-4}$, and 0.1 ml of this dilution was inoculated into each of the 3 tubes of streptomycin solution and into each of the 3 tubes of sterile distilled water. Incubation was carried out at 37°C.

Two similar sets of tubes were prepared and incubated at 5°C and at 22°C, respectively.

The results are shown in Graph 2. No multiplication occurred at 5°C either in the aqueous solution of streptomycin or in distilled water. At both 22° C and 37°C however, heavy growth occurred in the aqueous solution of streptomycin. Whereas the rate of growth was somewhat slower than had previously been observed when the organism was grown in a broth solution of streptomycin (Graph 1), the number of organisms per ml after 24 hours incubation was as great in the aqueous solution of streptomycin as it previously had been in the broth solution of streptomycin. At 22°C and at 37°C, slight multiplication took place in the distilled water. The amount of growth occurring in the water alone was, however, far less than in the aqueous solution of streptomycin, and it is apparent, therefore, that this organism is capable of utilizing streptomycin for growth.

*Pathogenicity.* Mice of the Rockland regular strain inoculated intraperitoneally with an undiluted 4-hour plain broth culture died within less than 72 hours; mice similarly inoculated with dilutions of $10^{-1}$ - $10^{-7}$ all survived, indicating that the organism possesses little virulence for mice.

It has been observed by Miller and Bohnhoff that streptomycin-dependent strains of meningococci are virulent for mice only if the animals are treated simultaneously with streptomycin. The virulence of this strain, therefore, was tested in mice receiving 4 mg of streptomycin daily by the subcutaneous route. Again only those animals receiving undiluted culture failed to survive.

When heat-killed suspens[illegible]rganisms, grown on broth or agar, were injected intravenously into each of 3 rabbits, all of the animals showed marked pyrogenic responses consisting of temperature rises ranging from 1.2 to 2.2°C. These animals appeared ill soon after injection and 7 of the 9 died within 24 hours.

Seven-tenths of a ml of each of these suspensions, when administered intravenously to mice (Swiss strain), produced no reaction. It seems possible, therefore, that death of the rabbits receiving this material may have been due primarily to pyrogenic reactions rather than to the presence of other toxic substances.

All animals receiving the aqueous suspension of standard pyrogen prepared from *Ps. pyocyaneus*‡ survived with no untoward reaction other than rise in temperature.

*Discussion.* The isolation of an organism which is not only resistant to the antibacterial action of streptomycin but capable of multiplying in the presence of streptomycin as the sole source of nutrient has been described. Recently a second organism has been isolated which is also resistant to streptomycin and capable of growing in high concentrations of the drug. This organism is a slender gram-negative rod which grows on beef infusion agar in the form of an opaque creamy yellow colony, 2-3 mm in diameter. On blood agar it produces a grayish-red pigment after 1 to 2 weeks at room temperature. Sugars are not fermented readily.

The fact that such organisms may exist is significant. It is now apparent that microorganisms may be divided into 4 general categories, in relation to streptomycin: (1) the streptomycin-sensitive cells, including those that are capable of growing in culture media without streptomycin but are inhibited by the presence of streptomycin, (2) the streptomycin-resistant cells, which are capable of growing either in culture media alone or in media containing streptomycin, (3) streptomycin-resistant cells, which are capable of multiplying in culture media, in media containing streptomycin, or in aqueous solutions of streptomycin, and (4) streptomycin-dependent

‡ Prepared from a culture of *Ps. pyocyaneus* according to the method of Welch, Calvery, McClosky, and Price[4] and subsequently purified further by removal of protein.

4 Welch, H., Calvery, H. O., McClosky, W. T., and Price, C. W., *J. Am. Pharm. Assn.*, 1943, **32**, 65.

...e in-
...s.
...Radioac-
...ported upon
...who administered
... a group of 6 cotton
rats ... *Litomosoides carinii*, sacrificing their animals 24 hours after intraperitoneal injection; by DuPont *et al.*,[5] who injected radio-arsenic intravenously in 37 rabbits; and by Hunter and co-workers,[6] who studied distribution in tissues, and in various chemical fractions of tissue, of radio-arsenic administered subcutaneously to rats, rabbits, guinea pigs, higher apes, and man. All of these experiments showed a remarkable degree of individual variation among animals of the same species receiving apparently identical treatment.

...The short half-lif... tracer experiments of long duration impossible; but the rapid decay is useful in therapeutic applications, as radiation dosage is easily controlled.

In order to calculate radiation dosage from any radioactive material to a particular tissue, it is essential to know not only the physical characteristics of the isotope employed, but also the concentration of the material in the tissue under consideration. For this reason, as well as for the more theoretical purposes discussed in an earlier paper,[1] we began a series of studies on the fate of radio-arsenic in laboratory animals, and, later, in man. Several phases of this research still are far from complete; however, because of the therapeutic potentialities of arsenic[76],[2] and because radioisotopes of arsenic are now offered for general distribution,[3] we feel that our studies on dis-

In general, all animals in all experiments exhibited greatest arsenic concentration in liver, kidney, spleen, and lung. Low arsenic uptake by the Brown-Pearce rabbit tumor was found by the DuPont group, who also reported no change in the tissue distribution pattern of tumor-bearing animals. Hunter *et al.* noted a remarkable difference between

1 Straube, R. L., Neal, W. B., Jr., Kelly, T., and Ducoff, H. S., Part I. PROC. SOC. EXP. BIOL. AND MED., in press.

2 Neal, W. B., Jr., Jacobson, L. O., Brues, A. M., Ducoff, H. S., Straube, R. L., and Kelly, T., *Am. Assn. for Cancer Research*, March 13, 1948, Atlantic City, N. J.

3 U. S. Atomic Energy Commission, Radioisotopes—Catalogue and Price List, No. 2, September, 1947.

4 Lawton, A. H., Ness, A. T., Brady, F. V., and Cowie, D. B., *Science*, 1945, **102**, 120.

5 DuPont, O., Ariel, I., and Warren, S. L., *Am. J. Syph. Gon. and Ven. Dis.*, 1942, **26**, 96.

6 Hunter, F. T., Kip, A. F., and Irvine, J. W., Jr., *J. Pharm. and Exp. Therap.*, 1942, **76**, 207; Lowry, O. H., Hunter, F. T., Kip, A. F., and Irvine, J. W., Jr., *ibid.*, 1942, **76**, 221.

arsenic distribution in rats and in any other species studied: in rats, arsenic is concentrated to a great extent in the blood, particularly the erythrocytes; in other animals, arsenic does not remain long in the blood stream, but is rapidly distributed to the tissues.

*Technics.* Arsenic was administered as sodium arsenite in buffered isotonic solution; the preparation of the arsenic solution has been described.[1]

Mice were killed by breaking their necks, rats and rabbits by injection of nembutal. Groups of mice and of rats were usually sacrificed at one time, and necropsied as quickly as possible; at no time did more than 1½ hours elapse between sacrifice of an animal and necropsy. Tissues were placed in previously weighed containers and weighed immediately to minimize loss of water by evaporation; they were then wet-ashed, and the activity measured with a thin mica end-window Geiger-Mueller tube. Similar treatment was accorded to biopsy specimens from patients, and to tissue samples from one patient who was moribund at the time of administration.

The wet-ashing technic: Whole organs (except livers) of mice were placed directly in porcelain counting capsules; whole organs of rats, livers of mice, and portions of organs of rabbits and of man were first placed in small pyrex beakers. The material was dissolved in fuming nitric acid and evaporated to dryness in a hood under a battery of 300 watt Mazda Reflector flood lamps. Solutions in beakers were transferred to capsules as soon as their volumes were sufficiently reduced; this was followed by one rinsing of the beaker with acid and 2 rinsings with distilled water, the rinsings being added to the capsules. This procedure resulted in a layer thin enough for accurate counting, and the nitric acid prevented formation and consequent loss of $AsCl_3$.

Stool samples were placed in bakelite-capped glass specimen jars, and activity measured directly in a calibrated high pressure gamma ionization chamber using a vibrating reed galvanometer.[7]

TABLE I.

Arsenic[76] Excretion, Rats and Rabbits (in microcuries).

| Dose (μc/g) | Total dose = 47 μc/rat (urine only—no activity in feces) .024 | .022 | .023 | .027 | .027 | Total dose = 235 μc/rabbit (urine and feces) .072 | | .082 | | .076 | | .070 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hr after injection | | | | | | Urine | Feces | Urine | Feces | Urine | Feces | Urine | Feces |
| 0- 6 | | | | | | 23.0 | 0.6 | | | | | | |
| 0-24 | 2.74 | 3.19 | 6.85 | 2.36 | 3.92 | | | 150 | 10 | 140 | 13 | 131 | 11 |
| 24-48 | | | 0.92 | 0.61 | 0.46 | | | | | 23 | 2 | 35 | 4 |
| 48-72 | | | 0.68 | 0.36 | 0.40 | | | | | | | 15 | 2.5 |
| 72-96 | | | Tr | Tr | Tr | | | | | | | 15 | Tr |

TABLE II.
Excretion in Man.

| Patient<br>Diagnosis<br>Dose | G. A.<br>Hodgkin's Disease<br>3.0 millicuries | | | G. R.<br>Lymphatic leukemia<br>2.7 millicuries | | |
|---|---|---|---|---|---|---|
| | Urine ($\mu$c) | Feces ($\mu$c) | % of dose excreted | Urine ($\mu$c) | Feces ($\mu$c) | % of dose excreted |
| 1st 24 hr | 498 | 2.0 | 16.7 | 116 | | 4.3 |
| 2nd " " | 548 | 3.7 | 18.5 | 574 | | 21.5 |
| 3rd " " | 288 | | 9.6 | 194 | 5.6 | 7.4 |
| 4th " " | 149 | 14.6 | 5.4 | 234 | 6.8 | 8.6 |
| 5th " " | 260 | 3.6 | 8.8 | 296 | | 11.0 |
| 6th " " | 124 | | 4.1 | 183 | | 6.8 |
| 7th " " | 104 | 5.2 | 3.7 | | | |

Figures on activity in all samples of tissues and of excreta are corrected for decay to the time of injection. Thus, activity is always proportional to the true amount of the isotope present for animals treated with the same injection solution, and animals of different treatment groups may be compared on the basis of "per cent of injected dose."

*Excretion studies. Rats:* Five male Sprague-Dawley rats, injected in the tail vein with 47 $\mu$c (0.2 cc), were immediately placed in metabolism cages, and excreta were collected at 24 hour intervals. Activity in the fecal samples was too low to count; Table I shows the data on the urine samples.

*Rabbits*: Four stock rabbits each received 235 $\mu$c (1 cc) via ear vein. Excreta were collected at intervals of 24 hours, except in the case of rabbit No. 39, which was sacrificed 6 hours after injection. Data on rabbit excreta are included in Table I.

*Man*: Table II summarizes the data on arsenic excretion in 2 representative patients injected intravenously.

*Mice*: No exact measurements were made on excretion rates in mice, but surveys with a portable meter on mouse cages and mice injected intraperitoneally indicated that some 75% of the injected dose is excreted within the first 24 hours.

It can be seen from the tables, and from Fig. 1, which summarizes the data, that arsenic excretion takes place far more slowly in rats (<10% the first 48 hours) than in rabbits (70%) or man (30-45%). More significant, probably, is the rapidity with which the arsenic[76] content of rats comes to equilibrium, as illustrated by the leveling off of the excretion curve.

In all species studied, including the rat, the feces account for less than 10% of the total arsenic excreted.

*Tissue distribution patterns. Rats*: Eight Sprague-Dawley males injected intravenously with 47 $\mu$c each were sacrificed in pairs at 6, 24, 48, and 96 hours. The results are shown in Table III.

The high concentration of arsenic in the

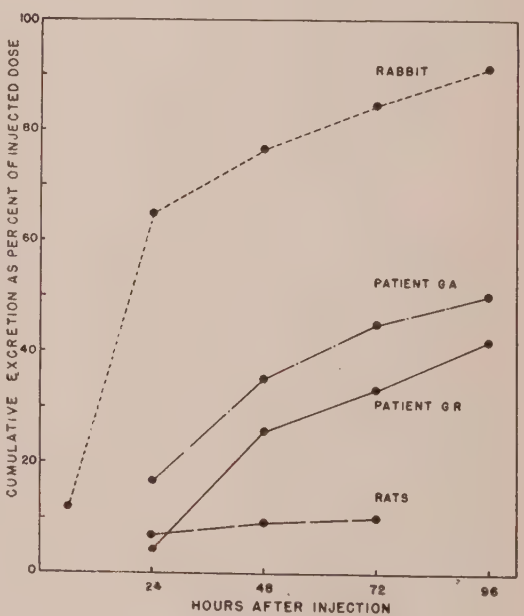



FIG. 1.
Excretion of Arsenic[76]. Cumulative excretion from the time of injection is expressed as percentage of the administered dose.

[7] Clemens, V., and Brar, S., CH-3830, Quarterly Report, Biology Division, June 1, 1947. Argonne National Laboratory.

TABLE III.
Tissue Distribution, Rats (mμc/g).

| Sacrificed at | 6 hr | | 24 hr | | 48 hr | | 96 hr | |
|---|---|---|---|---|---|---|---|---|
| Dose / Organ | 230 | 295 | 336 | 324 | 174 | 313 | 350 | 255 |
| Blood | 1705 | 2360 | 2780 | 2820 | 2600 | 2160 | 1390 | 1990 |
| Spleen | 603 | 662 | 990 | 1420 | 448 | 900 | 540 | |
| Heart | 538 | 628 | 700 | 840 | 239 | 266 | 328 | 365 |
| Lung | 442 | 420 | 542 | 598 | 512 | 356 | 610 | 460 |
| Kidney | 396 | 345 | 382 | 347 | 184 | 255 | 157 | 186 |
| Adrenal | 283 | | 255 | 323 | 298 | 213 | | 300 |
| Thymus | 200 | 407 | 142 | 388 | 186 | 304 | 630 | 254 |
| Liver | 174 | | 365 | | 148 | 159 | 158 | 104 |
| Testis | 43 | 65 | 74 | 80 | 22 | 41 | | 28 |
| Muscle | 58 | 50 | 41 | 42 | 11 | 23 | 22 | 12 |
| Skin and fur | 47 | | 64 | 70 | | | 38 | 78 |

TABLE IV.
Distribution of Arsenic in Tissues of Rabbits (mμc/g).

| Sacrifice time (hr after inj.) | 6 | 24 | 48 | 96 |
|---|---|---|---|---|
| Sex | ♀ | ♂ | ♂ | ♀ |
| Dose | 72 | 82 | 76 | 70 |
| Blood | 23 | 9 | 3 | |
| Spleen | 63 | 22 | 19 | |
| Heart | 46 | 13 | 3 | 3 |
| Lung | 104 | 72 | | |
| Kidney | 168 | 46 | 15 | |
| Liver | 415 | 62 | | 24 |
| Muscle | 36 | 27 | 10 | 5 |
| Femoral marrow | | 186 | 39 | 35 |
| Brain | 12 | 10 | 2 | |

blood is very striking. The fairly high arsenic content of spleen and lung, the large individual variation, and the lack of any great tendency for a decline in the arsenic content of most tissues, even after 48 hours, are also noteworthy. Inspection suggests that the high spleen values may be explained by the blood contained therein.

*Rabbits*: Table IV presents the data on tissue localization in the 4 rabbits used in the excretion studies (above). This pattern is in distinct contrast to that found with rats, in that concentrations of arsenic in blood after any time interval studied are never as great as in most of the solid tissues, and all tissues show a distinct reduction in arsenic content as time progresses. Liver, kidney, and lung contain the highest concentrations of arsenic.

*Mice:* Tissue localization of arsenic was studied in several series of mice, some bearing transplantable tumors. Tumors used were:

1. The Jackson-Brues embryoma,*[8] which is grown in C3H mice, develops slowly, and is quite variable as to rate of growth, percentage of "takes," and tissue organization; and

2. A lymphoma† which is grown in A mice, kills the host 4 to 6 weeks after transplantation, has 100% "takes," and is quite homogeneous.

Arsenic concentration was determined, as a rule, only in kidney, liver, lung, spleen, muscle, and tumor, when present. In all experiments, mice were injected intraperitoneally.

In the first study on mice 19 males of the A strain, 11 bearing lymphomas that had been transplanted 3 weeks previously, received 0.8 μc (in 0.25 ml) each. Table V records the results obtained in this experiment. Although this is a highly inbred strain, and although all efforts were made to treat the mice identically, the degree of individual variation within any

* Obtained from Dr. J. C. Aub of Harvard University.

[8] Jackson, E. B., and Brues, A. M., *Cancer Research*, 1941, **1**, 494.

† Kindly supplied by Dr. Egon Lorenz.

TABLE V.
Distribution of Arsenic in Tissues of "A" Mice.

| Sacrificed | Tumor, + or 0 | Dose, mμc/g | Concentration mμc As/g tissue | | | | |
|---|---|---|---|---|---|---|---|
| | | | Kidney | Liver | Spleen | Tumor | Muscle |
| At 6 hrs | 0 | 348 | 389 | 263 | 193 | | 343 |
| | 0 | 334 | 286 | 202 | 154 | | 85 |
| | + | 297 | 249 | 215 | 172 | 109 | 96 |
| | + | 276 | | 218 | | 144 | 188 |
| | + | 334 | 175 | 157 | 120 | 86 | 91 |
| " 12 " | 0 | 308 | 65 | 74 | 104 | | 85 |
| | 0 | 320 | 63 | 70 | 85 | | 82 |
| | + | 286 | 115 | 106 | 76 | 63 | 51 |
| | + | 268 | 87 | 92 | 54 | 33 | 48 |
| | + | 297 | 62 | 83 | 61 | 31 | 45 |
| " 24 " | 0 | 297 | 25* | 30 | 23* | | — |
| | 0 | 320 | 31 | 28 | — | | — |
| | + | 297 | 23* | 25 | 23* | 12 | 23* |
| | + | 268 | 25* | 37 | — | 11 | — |
| " 48 " | 0 | 348 | — | 10 | — | | — |
| | 0 | 334 | 26* | 14 | — | | — |
| | + | 334 | — | 21 | 41 | 23 | — |
| | + | 334 | — | 20 | — | 7.6 | — |
| | + | 348 | — | 27 | 32* | 8 8 | — |

— = Gave count of less than 1½ times background.
* = Based on count between 1½ and twice background.
Figures on activity and concentration have been corrected for decay to the time of injection.
1 mμc = 0.003 μg.

sacrifice group is extremely high.

It was noted, however, that the ratio of arsenic concentrations in kidney, liver, and spleen among non-tumorous mice of the early sacrifice groups appeared to be fairly constant. Accordingly, the per cent ratios of kidney, liver, and spleen concentrations were calculated as shown below:

Sample Calculation.
Kidney-liver-spleen ratio, first animal in Table V.

| Organ | Arsenic in 1 g tissue | Absolute ratio | % ratio |
|---|---|---|---|
| Kidney | 389 mμc | $\frac{389}{193} = 2.02$ | $\frac{389}{845} = 46\%$ |
| Liver | 263 mμc | $\frac{263}{193} = 1.36$ | $\frac{263}{845} = 31\%$ |
| Spleen | 193 mμc | $\frac{193}{193} = 1.00$ | $\frac{193}{845} = 23\%$ |
| Sum | 845 | 2.0:1.4:1.0 | 46-31-23 |

The calculated percentage ratios for these mice, and for a group of female C3H mice, some bearing transplanted embryomas, which were injected intraperitoneally with 6.7 μc, are shown in Table VI. It is readily seen that though there is greater variation in the kidney-liver-spleen (KLS) ratios for this group of C3H mice, the variation both from tumorless animals and from each other among the embryoma-bearers is quite striking.

The existence of a reproducible ratio of concentration between various tissues of normal mice, despite the remarkably wide range of variation in absolute values, may be explained, at least partially, on the following basis: The rapid rate of arsenic excretion by rabbits, mice, and man has been demonstrated. Any variation in excretion rate will therefore be greatly exaggerated unless concentration is expressed as percentage of retained, rather than total dose; *e.g.*, a variation of 10% in the quantity excreted is equivalent to a variation of some 40% in the dose retained.

It should be noted, however, that rats, with a very low rate of excretion, show as high a degree of variation in concentration patterns as any other animal studied. This may be partly accounted for by variability in blood content of organs.

TABLE VI.
Kidney-Liver-Spleen Ratios in Mice.

| Strain | Hr after inj. | Proportion (% basis) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Non-tumorous | | | Tumor-bearing | | |
| | | Kidney | Liver | Spleen | Kidney | Liver | Spleen |
| A | 6 | 46 | 31 | 23 | 39 | 34 | 27 |
| | | 45 | 32 | 24 | 38 | 35 | 27 |
| A | 12 | 29 | 31 | 40 | 31 | 41 | 29 |
| | | 28 | 32 | 40 | 38 | 36 | 26 |
| | | | | | 37 | 40 | 23 |
| $C_3H$ | 12 | 22 | 42 | 36 | 41 | 35 | 24 |
| | | 21 | 47 | 32 | 34 | 42 | 24 |
| | | 26 | 47 | 27 | | | |
| | | 32 | 36 | 33 | | | |
| | | 21 | 38 | 41 | | | |
| | | 24 | 44 | 32 | | | |
| | | 31 | 43 | 27 | | | |
| | | 27 | 40 | 33 | | | |
| $C_3H$ | 24 | 21 | 34 | 46 | 49 | 36 | 15 |
| | | 20 | 40 | 41 | 18 | 45 | 36 |
| | | 25 | 34 | 40 | | | |
| | | 17 | 26 | 57 | | | |

TABLE VII.
Tissue Distribution in Man (Patient H. N.).
(20 hours after injection.)

| Tissue | mμc/g |
|---|---|
| Liver | 46.4 |
| Kidney | 29.5 |
| Spleen | 16.1 |
| Parotid tumor | 15.6 |
| Heart | 14.6 |
| Jejunum | 14.3 |
| Vertebral marrow | 14.2 |
| Mesenteric lymph node | 12.8 |
| Stomach | 11.7 |
| Pancreas | 11.6 |
| Muscle (quadriceps) | 11.4 |
| Ileum | 11.1 |
| Lung | 10.8 |
| Femoral marrow | 10.8 |
| Adrenal | 8.5 |
| Ovary | 8.3 |
| Thyroid | 7.6 |
| Skin | 6.7 |
| Brain | 2.5 |
| Femoral cortical bone | 2.4 |

*Man:* A moribund 65-year-old female with carcinoma of the parotid was given 500 microcuries of $As^{76}$ (=4 mg arsenic) 20 hours before death. The distribution of activity in the tissues at time of death is shown in Table VII.

*Arsenic levels in the blood.* At the time of sacrifice of rabbits and of rats for distribution studies, specimens of blood were usually taken. Blood samples have also been obtained from patients, and a series of samples was drawn from one chicken. The results of these determinations are plotted semi-logarithmically as a function of time after injection in Fig. 2. The great degree of arsenic retention in rat blood is illustrated both by the high level at any particular time, and by the low negative slope of the curve.

*Discussion.* The change in distribution of arsenic in non-tumorous organs of tumor-bearing mice appears to be an example of a systemic effect wrought by a (histologically) localized phenomenon. Arsenic is largely bound to protein,[6] and, though to a lesser extent, to the –SH groups in cystine, glutathione, etc.[9] The proportion of arsenic retained in a particular organ can presumably be altered by

a. a change in the concentration of the arsenic binding constituents.

b. an alteration in the arsenic-combining capacity of some chemical substance, or

c. a combination of a and b.

Although the presence of tumor clearly alters the distribution of arsenic, the direction of the change in a particular organ (as, say,

[9] Voegtlin, C., Dyer, H. A., and Leonard, C. S., *Public Health Reports*, 1923, **38**, 1882.

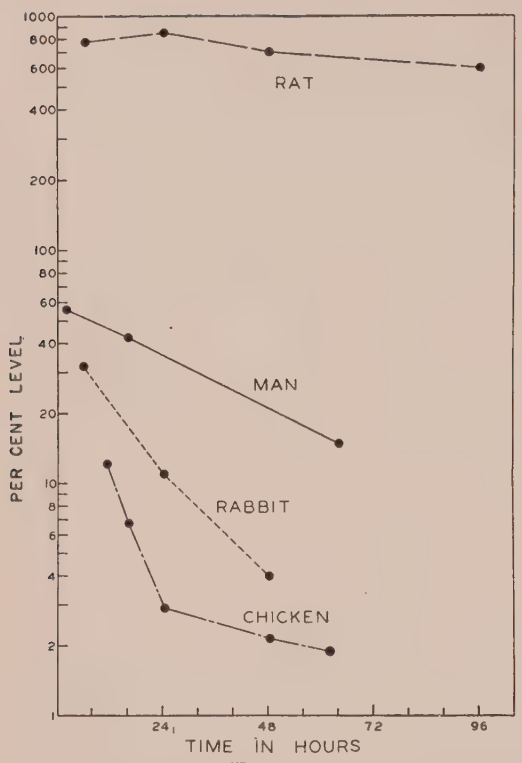



FIG. 2.

Arsenic[76] levels in whole blood. Concentration of arsenic[76] per gram of blood at a particular time is expressed as percentage of the administered dose per gram of body weight.

liver) is unpredictable.

Several workers have considered the effects of tumors on chemical composition of uninvolved organs, and the question has been reviewed in part by Toennies.[10] Glutathione and cystine appear to have been especially altered in unaffected organs of tumorous animals, as reported by Voegtlin and Thompson, by Woodward, and by Schenk.[11] It should also be noted that changes in the –SH levels of blood plasma are found in the presence of a great variety of tumors, and form the basis for several attempts at formulating serodiagnostic tests for cancer.[12]

We have as yet been unable to find any unique physiological or chemical property of rat's blood which might explain the high degree of arsenic retention. The deceleration of excretion rate beyond the first day makes it appear that retention in the rat is not primarily due to inability to excrete arsenic; the high blood concentration with preference for erythrocytes[6] suggests that the red cell of the rat may contain a system binding arsenic.

*Summary.* 1. Arsenic excretion was studied in man, rats, and rabbits. Less than 10% of the excreted arsenic is found in feces in any of these species; rats have by far the slowest rate of excretion.

2. Data are given for arsenic distribution in various organs in man, the rat, the rabbit, and 2 strains of mouse.

3. The degree of individual variation within each species was very great; in contrast to man and to other animals studied, the rat retains most of the injected dose in the blood for a considerable length of time.

4. The ratio of arsenic concentration in kidney, liver, and spleen of healthy inbred mice was found to be fairly constant for a given time after administration, and this ratio is suggested as a criterion for effects of various types of treatment.

5. Using this ratio as criterion, it was found that arsenic distribution is altered by the presence of transplanted tumors.

6. Factors changing arsenic distribution are discussed in relation to effects on levels of sulfhydryl-containing substances.

Acknowledgment is here made of the valuable assistance of Dr. Henry Hopple, Vladimir Clemens and Sarmukh Brar.

10 Toennies, G., *Cancer Research,* 1947, **7**, 193.

11 Voegtlin, C., and Thompson, J. W., *J. Biol. Chem.,* 1926, **70**, 801; Woodward, G. E., *Biochem. J.,* 1935, **29**, 2405; Schenck, E. C., *Arch. f. exp. Path. u. Pharm.,* 1934, **175**, 405.

12 Brdicka, R., *Nature,* 1937, **139**, 330; Walker, A. C., and Reimann, S. P., *Am. J. Cancer,* 1939, **37**, 585; Winzler, R. J., and Burk, D., *J. Nat. Cancer Inst.,* 1944, **4**, 417.

TABLE I.
Inhibition of Sheep Cell Agglutinins in Infectious Mononucleosis Serum by Beef Stroma Heated for 30 Minutes.

| Temperature °C | Beef stroma dilutions | | | |
|---|---|---|---|---|
| | 16,000 | 32,000 | 64,000 | 128,000 |
| 40 | — | — | — | ++++ |
| 60 | — | — | — | ++++ |
| 80 | — | — | + | ++++ |
| 100 | — | — | ++ | ++++ |

TABLE II.
Inhibition of Sheep Cell Agglutinins in Infectious Mononucleosis Serum by Beef Stroma Digested by Trypsin or Pepsin.

| Treatment of the beef stroma | Beef stroma dilutions |
|---|---|
| Pepsin at pH 3.0 2 days 37°C | 1:128,000 |
| " " " " 3 " " | 1:128,000 |
| Control " " " 3 " " | 1:128,000 |
| Trypsin at pH 7.7 2 days 37°C | 1:32,000 |
| " " " " 5 " " | 1:64,000 |
| Control " " " 5 " " | 1:64,000 |

TABLE III.
Inhibition of Sheep Cell Agglutinins in Infectious Mononucleosis Serum by Beef Stroma Extracted with Organic Solvents.

| Extraction | Extracted substance in % | Inhibition in dilutions | |
|---|---|---|---|
| | | Extracted stroma | Extract |
| Acetone, room temperature | 8.65 | 1:64,000 | 0 |
| Alcohol, " " | 20.0 | 1:16,000 | 1: 250 |
| Alcohol, after acetone extraction, room temperature | 12.6 | 1:16,000 | 1: 125 |
| Ether, boiling | 14.3 | 1:64,000 | 1:8000 |
| Acetone, " | 15.8 | 1:64,000 | 0 |
| Pyridine, 90°C, after acetone- and alcohol-extraction, at room temperature | 4.9 | 1: 8,000 | 1:2000 |

and at the end of the experiment. From Table II it can be seen that neither trypsin nor pepsin digests the M.A. In view of these experiments, it appeared improbable that the M.A. of beef erythrocytes is a protein. Apart from the M.A., beef stroma also contains a rapidly digested protein-like antigen which will be reported in a later publication.

Attempts were made to extract the M.A. with cold and with boiling organic solvents. The extraction with cold liquids was carried out by stirring for several days, the extraction with boiling liquids by changing the solvent several times until the material was exhausted.

As can be seen from Table III, a small quantity of M.A. could be extracted by means of boiling ether or hot pyridine. However, since, contrary to the statements of Stuart, Griffin, Fulton, and Anderson,[1] the cold alcoholic extract also shows a small but definite activity, the possibilities of extraction by means of boiling ethyl alcohol were thoroughly investigated as described below. Beef stroma was extracted, first with acetone and then with cold 100% alcohol. On each occasion 50 cc solvent per g stroma were used and the suspension stirred for 2 days at room temperature.

In this way, we were able to remove about 20% almost inactive material. After this pretreatment with cold organic solvents the beef stroma was exhaustively extracted with boiling 100% alcohol under reflux. The solvent was changed several times and, in this way,

TABLE IV.
Inhibition of Sheep Cell Agglutinins in Infectious Mononucleosis Serum by Beef Stroma Extracted First with Acetone and Ethyl Alcohol at Room Temperature and Later with Boiling Ethyl Alcohol.

| Extracted with | Fraction | Activity of the extract |
|---|---|---|
| Acetone, room temperature | 0-4 days | 0 |
| Alcohol, " " 100% | 0-4 " | 1:125 |
| Alcohol, boiling, 100% | 0- 15 min. | 1:32,000 |
| " " " | 15- 30 " | 1:16,000 |
| " " " | 30- 60 " | 1: 8,000 |
| " " " | 60-120 " | 1: 4,000 |
| Alcohol, boiling, 80% | 0- 15 min. | 1:1,000,000 |
| " " " | 15- 30 " | 1: 250,000 |
| " " " | 30- 60 " | 1: 500,000 |
| " " " | 60-120 " | 1: 250,000 |
| " " " | 2-4 hr | 1: 500,000 |
| " " " | 4-8 " | 1: 32,000 |
| Stroma residue | | 1:8,000 |

TABLE V.
Purification of a Less Active Beef Stroma Extract. (Initial dry weight 1.7 g).

| Extraction | Extracted substance in % | Inhibition of Sheep cell agglutination |
|---|---|---|
| Boiling acetone, 170 cc 0-15 min. | 21.0 | 0 |
| " " " " 15-30 " | | |
| " " " " 30-60 " | | |
| Boiling 100% alcohol 85 cc 0- 15 min. | 16.2 | 1:4,000 |
| " " " " " 15- 30 " | 6.75 | 1:8,000 |
| " " " " " 30- 60 " | 2.0 | 1:8,000 |
| " " " " " 60-120 " | 1.6 | 1:8,000 |
| Boiling 80% alcohol 85 cc 0- 15 min. | 28.0 | 1:1,000,000 |
| " " " " " 15- 30 " | 4.55 | 1: 320,000 |
| " " " " " 30- 60 " | 0.7 | 1: 480,000 |
| " " " " " 60-120 " | 0.95 | 1: 320,000 |
| Residue | 29.2 | |

various fractions were obtained. After completion of the exhaustive extraction with 100% alcohol, which removed about 0.3-0.7% dry substance calculated with reference to the stroma, extraction under reflux was performed with 80% alcohol. The fraction so obtained contained the M.A. in a dissolved form and in very much higher activity than in the stroma itself.

Table IV shows that, using 100% alcohol, the M.A. isolated has only poor activity, but that, when 80% alcohol is used, the activity jumps to 1,000,000. Likewise, a drop in the activity of the stroma residue is manifest. Altogether 5.1% dry substance can be extracted from the stroma, with 80% boiling alcohol. This value shows a certain variation dependent upon the stroma preparation and upon the conditions of the experiment.

From numerous experiments we obtained fractions of high activity (1:256,000-1:1,000,000) and low activity (below 1:256,000), which we sometimes combined.

Further purification of the two fractions was achieved by the following procedure:

The alcohol was distilled off under reduced pressure the residue evaporated to dryness on the water bath and then exhaustively extracted under reflux, first with acetone, changing the solvent several times, then with 100% alcohol and finally with 80% alcohol.

In this way, a small quantity of highly active substance could be recovered from the fraction with low activity, an indication of the

efficiency of the method.

Table V shows that an activity of 1:1,000,000 can be reached starting from a mixed fraction possessing an initial activity of about 1:250,000.

After standing for one day at 4°C, the 80% alcohol extract with an activity of 1:1,000,000 gave a precipitate which was removed by centrifugation. The precipitate showed an activity of 1:80,000 and the material remaining in the solution an activity of 1:2,400,000. The last fraction, which was obtained as a brownish red powder after evaporating off the alcohol, was designated by us as a purified heterogenetic "mononucleosis antigen." It may be mentioned that we have attempted to extract the M.A. more readily from the stroma by saponification of the latter with sodium alcoholate (50 mg Na in 50 cc alcohol) under reflux. We found that this resulted in inactivation of the antigen.

We have attempted a further purification by treating the aqueous and the alcoholic solutions with activated charcoal both in the cold and at the boiling point. The charcoal effected complete decolorization, but, at the same time, the active substance also disappeared from the alcoholic solution. It could be detected in a suspension of the charcoal at a dilution of 1:8000 but could no longer be recovered.

Furthermore, we investigated the question whether M.A. is a necessary constituent of beef erythrocytes or whether as Sohier, Jaulmes, and Tissier[5] presume, there are individual variations. Apart from numerous tests with mixed blood, from which we were always able to obtain the antigen, we have examined blood from three animals separately. In each case, we were able to obtain the antigen with correspondingly good activity. A further source of M.A. is sheep's blood stroma. From this, using the extraction technic described above, we were only able to achieve activities of the alcoholic extract up to 1:8000.

Stuart, Griffin, Wheeler, and Battey[2] were able to demonstrate the presence of M.A. in rabbit serum by means of the hemolysis inhibition technic. Using our adsorption technic, we have examined dried rabbit organs: the heart, liver, spleen and cerebellum showed an activity of 1:250, the kidneys as much as 1:500, the stomach, small intestine, lungs and cerebrum only 1:64, and muscle was inactive. The heart, liver, spleen and kidneys were combined and extracted according to the scheme described. The cold acetone and alcohol extracts, as well as the boiling 100% alcohol fractions, were inactive. The boiling 80% alcohol extract exhibited an activity of 1:500 after 15 minutes and this rose to 1:4000 after extracting for 2 hours. As claimed by Stuart and his coworkers, the reason why the rabbit does not produce an antibody against M.A. fraction of beef erythrocytes is probably that it contains this antigen in its own organs. Thus, the heterogenetic M.A. occurs in beef and sheep erythrocytes as well as in rabbit organs. On the other hand, no trace of M.A. could be detected in rabbit erythrocytes, in peptone, in pepsin, or in trypsin preparations.

*Summary.* 1. From the stroma of beef erythrocytes it was possible to extract with boiling 80% alcohol the so-called heterogenetic "mononucleosis antigen," which, after purification, inhibits the sheep cell agglutination of the infectious mononucleosis serum in a dilution of 1:2,400,000. It is a thermostable hapten and is not digested by pepsin or trypsin.

2. The "mononucleosis antigen" which occurs in sheep stroma cannot be extracted with good activity by means of the technic effective for beef cells.

3. A serologically similar heterogenetic antigen could also be detected in rabbit organs, although in small quantities. This fact makes it comprehensible why the corresponding antibody cannot be artificially produced in rabbits.

5 Sohier, R., Jaulmes, Ch., and Tissier, M., *Ann. Inst. Pasteur*, 1945, **71**, 463.

16787

# Nature of the Heterogenetic Hapten Reacting with Hemagglutinins in Horse Serum Sickness.

J. Tomcsik and H. Schwarzweiss.

*From the Hygienic Institute, University of Basel.*

An increase in the sheep's blood hemagglutinins in horse serum sickness has been described by both Hanganutziu and Deicher. Paul and Bunnel have described a similar phenomenon in infectious mononucleosis. It has subsequently been reported in numerous publications that the hemagglutinins in serum sickness and in infectious mononucleosis correspond to two different heterophilic antibodies (*e.g.*, Davidsohn *et al.*,[1-3] Stuart *et al.*[4-6] Schiff[7] even referred to a serum sickness antibody and gave the name serum sickness antigen (S.S.A.) to the receptor which reacts with the hemagglutinin in serum sickness. For the sake of simplicity, this expression will be retained in the present publication, although it should be noted that the part played by this antigen in the genesis of serum sickness has been in no way confirmed.

According to our conception, it is justifiable to include in the group of heterogenetic antigens, in addition to the Forssman antigen, both S.S.A. and the erythrocyte-antigen reacting with the antibodies in infectious mononucleosis (M.A.) as separate antigens. However, this conception does not appear to be a general one. For example, Boyd[8] writes: "The Forssman antigen is probably not a definite chemical entity, but a serological conception, a collective term covering substances which, injected into rabbits produce sheep hemolysins. The original Forssman antigen was a concept somewhat more narrow." In contrast to this conception, we consider it more correct to retain the original precise definition of the Forssman antigen and to recognize the other heterogenetic antigens mentioned as different substances. However, this conception presupposes a mosaic-like structure of certain cell antigens, the individual antigens being separable from one another even when they are components of a large molecule. The purpose of this work was to separate the S.S.A. from the other heterogenetic antigens described above, even when they occur in the same cell, and to elucidate its nature.

The technics for carrying out the hemagglutination and the inhibition reactions were described in previous papers.[9,10] When the nature of the hemagglutination is not more precisely defined, sheep's blood agglutination is to be understood. By degree of activity, we understand the reciprocal value of the highest dilution of antigen, calculated in terms of dry substance, which is capable of completely suppressing the hemagglutinating power of two antigen units.

For the purpose of selecting a human serum to serve in the subsequent course of the work as a serological indicator for the study of the S.S.A., 26 human sera were examined. The shortest interval of time between injection of the horse serum and removal of a sample of blood was 7 days. In 16 cases, the serum was obtained from more or less severe cases of serum sickness with exanthema. In 17 cases, the agglutination titer was more than

1 Davidsohn, J., *J. Immunol.*, 1929, **16**, 259.

2 Davidsohn, J., *J. Immunol.*, 1930, **18**, 31.

3 Davidsohn, J., *J. Infect. Dis.*, 1933, **53**, 219.

4 Stuart, C. A., Tallman, J., and Brintzenhoff, E., *J. Immunol.*, 1935, **28**, 85.

5 Stuart, C. A., Griffin, A. M., Wheeler, K. M., and Battey, S., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 212.

6 Stuart, C. A., Fulton, Mc. D., Ash, R. P., and Gregory, K. K., *J. Infect. Dis.*, 1936, **59**, 65.

7 Schiff, F., *J. Immunol.*, 1937, **33**, 305.

8 Boyd, W. C., Fundamentals of Immunology, Interscience Publishers, 1947, 2nd ed., p. 141.

9 Tomcsik, J., and Schwarzweiss, H., *Schweiz. Z. Path. und Bakt.*, 1947, **10**, 407.

10 Schwarzweiss, H., and Tomcsik, J., Proc. Soc. Exp. Biol. and Med., accompanying paper.

TABLE I.
Inhibition of Sheep Agglutinins in Serum Sickness, Infectious Mononucleosis, and Forssman Serum by Various Agents.

| Antigens | Inhibition titer of the antigens toward hemagglutinins in | | |
|---|---|---|---|
| | Serum sickness | Inf. mononucl. | Forssman serum |
| Sheep erythrocyte stroma | 6,000 | 24,000 | 32,000 |
| Beef " " | 64,000 | 64,000 | 0 |
| Horse " " | 4,000 | 8,000 | 0 |
| " " | 120* | 320* | 0 |
| " serum | 4* | 0 | 0 |
| Rabbit erythrocyte stroma | 250 | 0 | 0 |
| " " | 500* | 0 | 0 |
| " " hemolyzed | trace | 0 | 0 |
| " spleen and kidney | 16,000 | 250 | 0 |
| " liver | 2,000 | 250 | 0 |
| " muscle | 2,000 | 0 | 0 |
| Guinea pig kidney | 4,000 | 0 | 1,200 |
| Alcoholic extr. of g. pig kidney | 4,000 | 0 | 16,000 |
| Several peptones | 0 | 0 | 0 |

0 = no inhibition in a dilution 1 : 62.5, calculated in terms of dry weight of stroma or of various organs, or in 10% suspension of erythrocytes.
* = the dilutions were calculated exceptionally in volumes and not in weight of the dry substance.

1:48, *i.e.*, 0.1 cc of the serum diluted 1:16 still completely agglutinated 0.2 cc of a 0.75% suspension of sheep red blood corpuscles. The type of hemagglutinin was determined by examining all the sera with various dilutions of the following antigens: 1. Sheep blood stroma. 2. Cattle blood stroma. 3. Guinea pig kidney. 4. Rabbit erythrocytes. The hemagglutinins were considered to be characteristic for horse serum sickness when they were adsorbed by all 4 antigens. Of 9 adult sera, which showed a high agglutinin titer, 7 sera were found to be typical for horse serum sickness on the basis of their adsorption, while of 8 sera obtained from children only 2 were found to be typical for horse serum sickness. The serum HD 85, which was selected as serological indicator for the subsequent work, agglutinated sheep red blood corpuscles at a dilution of 1:240, cattle blood corpuscles at 1:120, and hemolysed both types of blood corpuscles at a dilution of 1:120. The serological results summarized in Table I are the average values of several determinations.

In assessing the results shown in Table I it is to be noted that the difference between a negative and a positive agglutination was very easy to determine in the case of the infectious mononucleosis and the Forssman sera, whereas the transition from a negative to a positive reaction after adsorption of the serum sickness serum took place stepwise, with the result that the titer of the antigen with the latter serum exhibited fluctuations of more than 50% in different readings.

As can be seen from Table I, the beef stroma exhibited the highest serological activity of all antigens examined, both with mononucleosis and with serum sickness serum. Since boiling the aqueous stroma suspension is not detrimental to the serological activity, it may be concluded that the B.T. (beef thermostable substance) described by Stuart, Griffin, Wheeler, and Battey[5] contains both M.A. and S.S.A. On the other hand, as already known, guinea pig kidney contains the Forssman antigen and S.S.A. Thus, if our conception described above is correct, it should be possible to isolate S.S.A. both from beef stroma and from guinea pig kidney. Since we were able, as previously reported,[10] to isolate the heterogenic mononucleosis antigen in a purified form from B.T. by fractional extraction with cold and boiling organic solvents, we also employed the same procedure for the isolation of S.S.A.

Table II shows that we were able by means of 100% boiling alcohol to extract S.S.A. from beef stroma with a considerable increase in its activity. Since, as shown in a previous study,[10] a considerable increase in the M.A.

TABLE II.

Inhibition of Sheep Agglutinins in Infectious Mononucleosis and Serum Sickness Serum by Beef Stroma Extracted First with Acetone and Ethyl Alcohol at Room Temperature and Later with Boiling Ethyl Alcohol.

| Extracted with | Fraction | Inhibition titer of the extracts toward hemagglutinins in | |
|---|---|---|---|
| | | Inf. mononucl. | Serum sickness |
| Acetone, room temperature | 0- 4 days | 0 | 0 |
| Alcohol, " " 100% | 0- 4 " | 125 | 16,000 |
| Alcohol, boiling, 99.9% | 0- 4 hours | 16,000 | 192,000 |
| " " " | 4- 8 " | 48,000 | 256,000 |
| " " " | 8-12 " | 48,000 | 256,000 |
| Alcohol, boiling, 80% | 0- 4 hours | 1,100,000 | 6,000 |
| " " " | 4- 8 " | 500,000 | 48,000 |
| " " " | 8-12 " | 256,000 | 32,000 |

activity can be detected only in the fractions obtained with 80% boiling ethyl alcohol, it was possible to separate the two heterogenetic antigens from B.T. by means of a simple procedure based on their difference in solubility. After repeated application of this procedure, two fractions were obtained from beef stroma which exhibited the following activities:

Fraction A: S.S.A. activity 512,000, M.A. activity 8,000.

Fraction B: S.S.A. activity 16,000, M.A. activity 1,000,000.

Preparation of pure S.S.A. from rabbit stroma was not attempted as its activity was too small. It is all the more difficult to give an explanation of the small serological activity of this stroma in comparison with the intact or boiled rabbit erythrocyte suspension since the hemolysed erythrocytes were completely inactive; thus, the active substance of the stroma could not be detected outside the stroma.

The next question was whether the S.S.A. could be separated from the Forssman antigen of guinea pig kidney. 10 g dried and finely powdered guinea pig kidney were extracted twice, for 9 hr each time, with 250 cc 97% alcohol in a Soxhlet apparatus. Afterwards the residue was further extracted for varying lengths of time, up to 3 hours, with boiling 97% alcohol. Both antigens were detectable in the cold alcoholic extract, but when the already thoroughly extracted residue was further extracted with boiling alcohol, only S.S.A. with an activity of 1:1,000 could be detected. In contrast to beef stroma, it was thus possible to isolate the S.S.A. from guinea pig kidney, but without elevation of the serological activity.

After these investigations of its heterogenetic occurrence, S.S.A. was finally investigated in the original antigen, *i.e.*, in horse serum. 750 cc horse serum with a S.S.A. activity of 1:2 were coagulated at 100° and the coagulated mass of albumin separated from the liquid portion on a Buchner filter. Both fractions were dried in a vacuum drying oven. The suspension of coagulated albumin was still inactive against serum HD 85 in a dilution of 1:50, while the dried residual substance obtained from the filtrate showed an activity of 1:1,200. By fractional extraction with alcohol, however, no increase in activity could be achieved. Both the cold and the hot alcoholic extracts had a serological activity of about 1:1,500.

*Summary.* 1. From beef stroma pretreated at room temperature with acetone and alcohol, a fraction was isolated with boiling 100% alcohol which, in a dilution of 1:500,000, combines with the sheep blood agglutinin of human serum produced during serum sickness. Using the terminology of Schiff, this fraction corresponds to the heterogenetic serum sickness antigen; it could be separated to a large extent from the heterogenetic mononucleosis antigen which also occurs in beef stroma.

2. The so-called serum sickness antigen also

occurs heterogenetically in guinea pig kidney and it could also be isolated from the Forrsman antigen although without increase in activity.

3. Horse serum is a poorer source for isolation of the so-called serum sickness antigen. The latter could be isolated from the serum, after removal of the albumin bodies by coagulation with heat, with an activity of 1:1,500.

## 16788 P

## Dimethyl Ether of *d*-Tubocurarine Iodide as an Adjunct to Anesthesia.

V. K. STOELTING, J. P. GRAF, AND Z. VIEIRA. (Introduced by K. K. Chen.)

*From the Department of Anesthesiology, Indiana University School of Medicine, Indianapolis.*

The capacity of curare to block efferent nervous impulses at the myoneural junction has long been known. Bennett[1] administered curare clinically to minimize the trauma to patients undergoing convulsive shock therapy. Subsequently, Griffith and Johnson,[2] and Cullen[3] reported the use of curare as an adjuvant for relaxing the abdominal muscles during inhalation anesthesia.

The dosage of *d*-tubocurarine chloride administered to patients during anesthesia rarely produces toxic cerebral or cardiovascular manifestations. The drug frequently produces moderate or severe respiratory depression. For this reason, other natural and synthetic compounds have been investigated. One of the more promising preparations is dimethyl ether of *d*-tubocurarine iodide.[4,5] This compound has a chemical formula $C_{40}H_{48}O_6N_2I_2 \cdot 3H_2O$. It will be referred to as M-curare. In animal experiments, Chen and Swanson[6] demonstrated that M-curare effectively decreases muscle tone without producing respiratory depression.

*Experimental.* We have administered M-curare to anesthetized patients of both sexes, ranging in age from 10 to 84 years of age. The degree of relaxation reported was based on the evaluation of the surgeon.

TABLE I.

| Anesthesia | No. of patients | Range of initial dosage in mg | | |
|---|---|---|---|---|
| | | Min. | Max. | Avg |
| Cyclopropane | 16 | 1 | 4 | 2 |
| Ether | 49 | 1 | 5 | 2.5 |
| Nitrous oxide | 35 | 1 | 6.5 | 3 |

The drug was dissolved in a distilled water and the curare adjusted to .5 mg per cc.* A preservative was added. The pH varied from 4.0 to 5.0. The solutions were stored at room temperature and used over a period of several weeks. Different lots of the drug were administered by the intravenous route. The initial dose consisted of 1 mg or more of the drug.

Data presentede in the table show the amount of M-curare required to produce adequate relaxation. The dosage recorded was given in one or more injections within a period of 10 minutes. Adequate relaxation was not noted in any patients who received less than 1 mg of M-curare.

It will be noted that an average of 2 mg of M-curare produced satisfactory relaxation in 16 patients receiving cyclopropane anesthesia. An average of 2.25 mg was required to produce adequate relaxation with ether

1 Bennett, A. E., *Am. J. Med. Science,* 1941, **202**, 101.

2 Griffith, H. R., and Johnson, G. E., *Anesthesiology,* 1942, **3**, 418.

3 Cullen, S. C., *Surgery,* 1943, **14**, 261.

4 King, H., *J. Chem. Soc.*, London, 1935, (pt. 2), 1381-1389.

5 Collier, H. O., and Paris, S. K., *Nature,* 1948, **161**, 817.

6 Chen, K. K., and Swanson, E. E., personal communication of unpublished data.

* Supplied by Eli Lilly Research Laboratories, Indianapolis, Ind.

anesthesia. With nitrous oxide anesthesia, the comparable dosage was 3 mg.

Relaxation produced by the initial dose of M-curare sufficed for surgical procedures, lasting 60 to 90 minutes. After this period, supplemental injections of .5 to 1 mg were infrequently required. The anesthesia level was maintained in lower plane I or upper plane II in all the patients. Reflexes were absent in 8 patients at the termination of surgery. Intubation was performed in 44 patients where the type of surgery made the technic mandatory.

No cardiovascular changes were noted in any of the patients. In no case could a fall in blood pressure be attributed to the drug.

Mild respiratory depression was observed in 9 patients in a series of 100 cases. In 7 of these, respiratory depression existed before surgery as the result of excessive premedication. One of the remaining cases received 7.5 mg of M-curare during a 15-minute period. Five minutes after the final injection, the rate of respiration decreased from a normal of 22 per minute to 14. Ten minutes later it had returned to the normal rate. Subsequently, the patient received injections of .5 mg and 1 mg without the development of respiratory embarrassment. Mild respiratory depression occurred in the other patient 3 minutes after the injection of 1.5 mg of M-curare. The rate of respiration decreased from a normal of 24 per minute to 16. This depression persisted for 5 minutes and the rate of respiration returned to a normal of 24. Subsequent injections of .5 mg and 1 mg of M-curare caused no untoward respiratory changes. Each of these patients maintained an adequate tidal exchange during the period of respiratory depression.

*Discussion.* The relaxation obtained with M-curare in this study was comparable to that obtained by other workers using *d*-tubocurarine chloride. The drug appears to have a selective action on skeletal muscle similar to that of *d*-tubocurarine chloride, but seldom affects the muscles of respiration.

No gross or microscopic changes were observed by Chen and Swanson[6] in any of the organs of the experimental animals which received lethal doses of M-curare. The median lethal dose ($LD_{50}$) of M-curare in rabbits by intravenous injection, plus and minus standard error, was found to be $0.031 \pm 0.002$ mg per kg of body weight. The average dose sufficient to produce head drop in rabbits was approximately 0.0167 mg per kg of body weight (usually referred to as a rabbit unit). A group of 8 rabbits tolerated one rabbit unit per day for 10 days. At the end of this period, 6 out of 8 rabbits succumbed to an $LD_{50}$ injected intravenously. This gave evidence that neither tolerance nor cumulation had developed.

*Summary.* The administration of the dimethyl ether of *d*-tubocurarine iodide (M-curare) to 100 patients produced relaxation comparable to that of *d*-tubocurarine chloride. Respiratory depression was seldom observed and was of minor degree when present. Less M-curare than *d*-tubocurarine chloride is needed on a mg-for-mg basis.

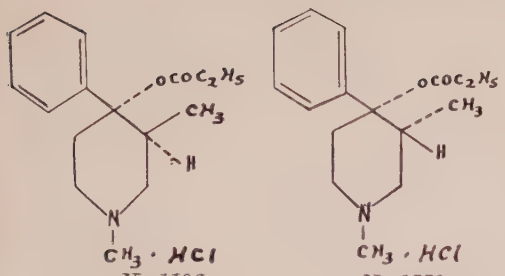
16789

## Effect of Two New Analgetic Agents on the Oxygen Consumption of Brain *In vitro*.

F. W. SCHUELER AND E. G. GROSS

*From the Department of Pharmacology, College of Medicine, The State University of Iowa.*

Quastel and others[1,2,3] have conducted investigations on the actions of hypnotics and anesthetic agents on brain metabolism in which they have indicated parallels to exist between *in vivo* and *in vitro* activity. Similar studies by Elliott, Warrens, and James[4] suggest that, except for their findings on succinate oxidation, there is no evidence that morphine,




Nu-1196 Nu-1779

FIG. 1.

demerol, and methadon exert their effects on oxidative processes in a manner similar to the anesthetics and hypnotics.

In the following investigation two new analgetic agents,[5] namely, (a) *dl*-α-1,3-dimethyl-4-phenyl-4-propionoxy-piperidine hydrochloride (Nu-1196), and (b) *dl*-β-1,3-dimethyl-4-phenyl-4-propionoxy-piperidine hydrochloride (Nu-1779)* were compared with methadon in regard to their *in vitro* effects on brain metabolism using the conventional manometric methods.[6,7] The formulae for these compounds are given in Fig. 1.

Randall and Lehman[8] have made studies on these compounds relative to analgesia in the rat. They report that Nu-1196 has about the same analgetic potency as morphine and Nu-1779 is about 5 times as active as morphine in rats. Studies[9] carried out in our laboratory indicate both of these new agents to be potent analgetics in normal subjects. Nu-1196, while being the least potent on a milligram basis, shows the greatest clinical promise since at comparable analgetic levels its side effects are minimal relative to Nu-1779 and morphine.

*Procedure and Results.* The oxygen consumption of brain cortex tissue slices (Rat) respiring in the presence of various substrates together with the three drugs Nu-1196, Nu-1779, and methadon were determined by the direct method of Warburg.

Cerebral cortex slices were cut with a razor and template[10] and the tissues were suspended in a modified Krebs-Ringer solution. The modified medium used was prepared according to the directions given by Elliott *et al.*[4] Phosphate buffer was used in all of the oxygen uptake measurements together with a gas phase of oxygen. After 2½ to 3 hours (*i.e.*, after at least 90% decrease in the initial $O_2$ uptake) the appropriate substrate was added. The shaking was continued for one hour after addition of the substrate. The drugs were made up in Ringer's solution and added to the tissues from the side arm after the control period of 60 minutes with substrate and continued for an additional 60-minute period. Each vessel, therefore, served as its own control and in addition controls were run for the entire 120-minute period.

Table I summarizes the experimental results expressed as per cent gross inhibition of oxygen consumption during the second hour over that obtained during the first hour. We have also summarized the oxygen uptake values in this table in terms of the $mm^3$ $O_2$ uptake per milligram of wet weight tissue per hour, due to the added substrate with and without drug. Table II summarizes the effect of the analgetic agents upon the oxygen uptake of brain after at least 90% decrease in the oxygen consumption where no substrate has been added. The per cent net inhibition of oxygen consumption due to drug alone was calculated by subtracting the per cent fall of the control during its second hour over its first hour from the respective per cent gross inhibitions due to the drugs.

1 Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, 1932, **26**, 725.

2 Jowett, M., *J. Physiol.*, 1938, **92**, 322.

3 Fuhrman, F. A., and Field, J., 2nd., *J. Pharm. and Exp. Therap.*, 1943, **77**, 392.

4 Elliott, H. W., Warrens, A. E., and James, H. P., *J. Pharm. and Exp. Therap.*, 1947, **91**, 98.

5 Ziering, A., and Lee, J., *J. Org. Chem.*, 1947, **12**, 911.

* These drugs were supplied by the Hoffmann-LaRoche, Inc., Nutley, N. J.

6 Dixon, MM., *Manometric Methods*, Macmillan Co., New York, 1943.

7 Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric Technics, Burgess Publ. Co., Minneapolis, Minn., 1945.

8 Randell, L. O., and Lehmann, G., *J. Pharm. and Exp. Therap.*, 1948, **93**, 314.

9 Gross, E. G., Holland, H. L., and Schueler, F. W., *J. Applied Physiol.*, in press.

10 Crimson, J. M., and Field, J., 2nd, *Am. J. Physiol.*, 1940, **130**, 231.

TABLE I.
Inhibition of the Oxygen Uptake of Brain Slices by Nu-1196 and Nu-1779.

| Substrate and drug | No. of flasks N | Δ (net change) in $O_2$ uptake due to the presence of the given substrate in the absence and in the presence of the drug: First hr* without drug | Second hr† with drug | % Gross inhibition $m \pm \sigma_m$ | % Net inhibition $M \pm \sigma_M$ |
|---|---|---|---|---|---|
| Glucose 0.2% | | | | | |
| Methadon .01M | 4 | 1.34 | 0.24 | 82.5 ± 1.2 | 74.7 ± 1.5 |
| Nu-1779 .01M | 8 | 1.43 | 0.88 | 48.0 ± 4.2 | 41.1 ± 4.4 |
| Nu-1196 .01M | 4 | 1.46 | 0.76 | 50.0 ± 5.5 | 42.2 ± 5.6 |
| Control (no drug) | 9 | 1.57 | 1.53 | 7.8 ± 1.1 | |
| Glucose 0.2% | | | | | |
| Morphine HCl .01M | 8 | 1.76 | 1.60 | 10.1 ± 5.2 | 2.1 ± 5.3 |
| Control (no drug) | 6 | 1.96 | 1.79 | 8.0 ± 1.2 | |
| Lactate 0.2% | | | | | |
| Methadon .01M | 11 | 0.40 | 0.31 | 68.7 ± 3.9 | 44.1 ± 5.9 |
| Nu-1779 .01M | 9 | 0.45 | 0.37 | 57.6 ± 6.3 | 33.0 ± 7.7 |
| Nu-1196 .01M | 12 | 0.44 | 0.24 | 51.0 ± 3.9 | 26.4 ± 5.9 |
| Control (no drug) | 9 | 0.44 | 0.41 | 24.6 ± 4.5 | |
| Succinate 0.2% | | | | | |
| Methadon .01M | 10 | 0.63 | 0.36 | 43.8 ± 3.2 | 19.5 ± 5.4 |
| Nu-1779 .01M | 10 | 0.62 | 0.49 | 36.9 ± 2.7 | 12.6 ± 5.1 |
| Nu-1196 .01M | 11 | 0.62 | 0.40 | 35.3 ± 2.5 | 11.0 ± 5.0 |
| Control (no drug) | 7 | 0.59 | 0.37 | 24.3 ± 4.3 | |
| Pyruvate 0.2% | | | | | |
| Methadon .01M | 7 | 0.47 | 0.11 | 74.9 ± 2.8 | 58.1 ± 5.3 |
| Nu-1779 .01M | 7 | 0.38 | 0.10 | 72.5 ± 2.9 | 55.7 ± 5.4 |
| Nu-1196 .01M | 7 | 0.36 | 0.11 | 69.3 ± 1.6 | 52.5 ± 4.8 |
| Control (no drug) | 6 | 0.36 | 0.30 | 16.8 ± 4.5 | |
| Citrate 0.2% | | | | | |
| Methadon .01M | 7 | 0.26 | 0.05 | 73.0 ± 1.3 | 39.0 ± 4.4 |
| Nu-1779 .01M | 6 | 0.20 | 0.03 | 86.2 ± 5.0 | 52.2 ± 6.5 |
| Nu-1196 .01M | 8 | 0.22 | 0.08 | 81.0 ± 2.1 | 47.0 ± 4.7 |
| Control (no drug) | 6 | 0.25 | 0.04 | 34.0 ± 4.2 | |
| α + β-glycerophosphate .2% (52% α) | | | | | |
| Methadon .01M | 4 | 0.40 | 0.32 | 49.0 ± 3.2 | 34.0 ± 7.3 |
| Nu-1779 .01M | 4 | 0.30 | 0.19 | 30.0 ± 8.8 | 15.0 ± 11.0 |
| Nu-1196 .01M | 4 | 0.34 | 0.17 | 18.2 ± 4.1 | 32.0 ± 7.7 |
| Control (no drug) | 4 | 0.34 | 0.26 | 15.0 ± 6.6 | |
| Glutamate .2% | | | | | |
| Methadon .01M | 4 | 0.28 | 0.07 | 58.0 ± 2.8 | 0.0 ± 4.2 |
| Nu-1779 .01M | 4 | 0.25 | 0.07 | 68.0 ± 1.9 | 10.0 ± 3.7 |
| Nu-1196 .01M | 4 | 0.25 | 0.10 | 74.0 ± 3.4 | 16.0 ± 4.7 |
| Control (no drug) | 4 | 0.28 | 0.11 | 58.0 ± 3.2 | |
| Oxalacetate .2% | | | | | |
| Methadon .01M | 4 | 0.35 | 0.14 | 62.5 ± 4.3 | — 0.5 ± 5.7 |
| Nu-1779 .01M | 4 | 0.32 | 0.23 | 44.0 ± 1.9 | —10.0 ± 4.1 |
| Nu-1196 .01M | 4 | 0.36 | 0.13 | 62.0 ± 1.8 | — 1.0 ± 4.2 |
| Control (no drug) | 4 | 0.33 | 0.12 | 63.0 ± 3.7 | |

m = Mean % gross inhibition.
$\sigma_m$ = Standard error of mean % gross inhibition.
M = Mean % net inhibition.
$\sigma_M$ = Standard error of mean % net inhibition.
M = m experiment — m control.

$\sigma_M = \sqrt{\sigma_m^2 \text{ experiment} + \sigma_m^2 \text{ control}}$

* Net average $mm^3$ $O_2$ uptake per hour per mg wet weight of tissue due to added substrate.
† Net average $mm^3$ $O_2$ uptake per hour per mg wet weight due to added substrate after addition of drug.

TABLE II.
Effect of Analgetic Agents on Oxygen Uptake of Brain After at Least 90% Decrease* in Respiration on Endogenous Substrates.

| | Average mm³ $O_2$ uptake per hr per mg wet weight of tissue | | | |
|---|---|---|---|---|
| | 1st hr | | 2nd hr | |
| | No drug. | No added substrate | With drug. | No added substrate |
| Morphine 0.01M | | .167 | | .019 |
| Methadon 0.01M | | .133 | | .029 |
| Nu-1779 0.01M | | .102 | | .042 |
| Nu-1196 0.01M | | .074 | | .011 |
| Control | | .161 | | .073 |

* Drug was added after 2½ - 3 hr of shaking in Warburg flasks. No substrate was added.

The effects produced by the drugs are compared at equi-molar concentrations with methadon (at 0.01M) on glucose as a reference substrate. This concentration of methadon was found experimentally to produce a significant inhibition of the oxygen consumption of brain respiring on glucose.[4]

*Conclusion.* Our experiments indicate that significant inhibitions of the oxygen consumption of brain slices may be obtained using the new analgetic agents Nu-1196 and Nu-1779 when this tissue respires on glucose, lactate, pyruvate, α- and β-glycerophosphate (52% α), citrate, and possibly succinate. It must be pointed out, however, that inhibitions due to drug with citrate as a substrate are to be considered quite dubious since the normal increase in $O_2$ consumption by this substrate alone is so small. Doubtful inhibitions were produced when brain slices respired on glutamate and oxalacetate. Nu-1196 and Nu-1779 showed insignificant effects at concentrations less than 0.01M.

As in the case of demerol, methadon, and morphine reported by Elliott *et al.*,[4] the concentrations of the new agents Nu-1196 and Nu-1779 required to produce significant inhibitions were vastly larger than that required in the production of *in vivo* effects.

16790

## Reduction in the Number of Adult *Trichinella spiralis* in Rats After Treatment with Naphthoquinones.*

JOSÉ OLIVER-GONZÁLEZ AND ERNEST BUEDING.

*From the Department of Pharmacology, Western Reserve University School of Medicine, Cleveland, O., and the School of Tropical Medicine, San Juan, Puerto Rico.*

The effect of naphthoquinones on the metabolism of *Schistosoma mansoni* has been studied recently.[1] 2-methyl-1,4-naphthoquinone inhibits the rate of glycolysis of *S. mansoni in vitro.* Administration of this compound with subcurative doses of "Fuadin" to mice infected with *S. mansoni* causes a marked decrease in the rate of glycolysis of the worms. Other naphthoquinones varied as to the degree of their anti-glycolytic activity *in vitro* as well as *in vivo.* The inhibitory effect on glycolysis manifested by these naphthoquinones is decreased markedly when the worms are incubated in dialysed blood serum instead of a protein-free salt solution. This suggests that interaction of the naphthoquinones with serum proteins would reduce any intrinsic activity of these compounds

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

1 Bueding, E., Peters, L., and Waite, J. F., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 111.

TABLE I.
Number of Adult Trichinae Recovered from Rats After Treatment with a Single Oral Dose of 200 mg of Various 1,4-quinones.

| Compound | No. of rats used | | Avg No. of worms recovered | |
|---|---|---|---|---|
| | Treated | Untreated | Treated | Untreated |
| 2-Methyl-1,4-naphthoquinone | 5 | 2 | 49 | 250 |
| ,, ,, | 5 | 2 | 217 | 689 |
| ,, ,, | 5 | 2 | 76 | 461 |
| ,, ,, | 10 | 3 | 39 | 411 |
| ,, ,, | 10 | 3 | 88 | 615 |
| 2-Hydroxy-3-N-piperidinomethyl-1,4-naphthoquinone | 2 | 2 | 100 | 518 |
| 2-Hydroxy-3-N-piperidinomethyl-1,4-naphthoquinone | 5 | 2 | 68 | 507 |
| 2-Hydroxy-3-N(2-methylpiperidino)-methyl-1,4-naphthoquinone | 2 | 2 | 483 | 467 |
| 3-Hydroxy-2-isoamyl-naphthoquinone | 2 | 2 | 412 | 510 |
| 3-Hydroxy-2-methyl-octyl-1,4-naphthoquinone | 2 | 2 | 361 | 366 |
| 2,3-Dichloro-1,4-naphthoquinone | 2 | | 508 | |
| Tolu-p-quinone | 2 | 2* | 581 | 604* |
| Beta-carbomethoxyethyl-(2-methyl-3-n-thiobutyl-1,4-naphthoquinonyl-6)-ketone | 2 | | 388 | |
| 2-Methyl-3-thioethyl-6-butyryl-1,4-naphthoquinone | 2 | | 444 | |
| 3-N-Thioamyl-2-chloro-1,4-naphthoquinone | 2 | 2* | 319 | 420* |
| 2,5-ditertiary-butyl-1,4-hydroquinone | 2 | | 480 | |

* The same two rats served as controls for the accompanying three drugs tested.

against parasites living in a habitat with a high protein content. Of interest, therefore, is the study not only of their therapeutic utility, but also of the effect of naphthoquinones on the metabolism of intestinal worms, since these live in a medium relatively low in protein. The following is a report of the results obtained after treating rats with naphthoquinones during the intestinal stage of infection with *Trichinella spiralis*.

Rats weighing from 200 to 300 g were fed by stomach tube with 1100 to 1500 infective trichinae larvae each. The larvae were obtained from trichinous rat meat digested in a pepsin-hydrochloric acid mixture. Twenty-four hours later the rats were given a single dose of 200 mg† of the compound to be tested.‡ The drug was suspended in mucilage of tragacanth and then fed through a stomach tube. On the fifth day after infection the rats were killed and the number of adult trichinae present in small and large intestine were recovered and counted. Untreated rats were killed together with the treated ones to serve as controls.

Of the 11 compounds tested only two (2-methyl-1,4-naphthoquinone and 2-hydroxy - 3 - piperidinomethyl - 1,4-naphthoquinone) caused a diminution in the number of intestinal trichinae (Table I). The average number of worms recovered in the animals treated with these two compounds was significantly less than in the untreated ones. The chi-square test was calculated for each drug and the values of $P$ were beyond the 1% level of significance.

It is of interest that the reduction in the number of worms was caused by only two of the naphthoquinones tested. Since all the compounds are closely related, the effect is apparently determined by highly specific groups attached to the naphthoquinone nu-

† For some of the compounds, 200 mg was the maximum tolerated dose for rats.

‡ The compounds used were supplied generously by Dr. Louis F. Fieser, Department of Chemistry, Harvard University, and by the Abbott Laboratories, North Chicago, Ill.

cleus. Several of the compounds that had no effect on adult *T. spiralis* are more effective inhibitors of glycolysis of *S. mansoni* than 2-methyl-1,4-naphthoquinone.[2] On the other hand, the anti-glycolytic activity of 2-hydroxy - 3 - piperidinomethyl - 1,4-naphthoquinone against glycolysis of schistosomes was a hundred times lower than that of 2-methyl-1,4-naphthoquinone.[1] Apparently, two different mechanisms are involved in the effect of naphthoquinones against adult trichinae and on glycolysis of schistosomes, since compounds that possess high anti-glycolytic activity did not reduce the number of trichinellae and the reverse was true for the 2-hydroxy-3-piperidinomethyl derivative.

No indication was obtained as to whether 2-methyl-1,4-naphthoquinone inhibits any specific metabolic reaction of adult trichinellae. The metabolism at the adult stage of this parasite differs markedly from that of the larvae. Unlike the latter,[3] adult trichinellae have an extremely low rate of oxygen uptake and do not contain any significant amount of glycogen. Furthermore, it was found that the adult worms do not utilize glucose present in the medium and do not produce any significant amounts of lactic acid or other acids when incubated in a salt medium containing glucose.

*Summary.* The oral administration of 2-methyl-1,4-naphthoquinone and 2-hydroxy-3 - piperidinomethyl - 1,4-naphthoquinone to rats infected with *Trichinella spiralis* produced a significant reduction in the number of intestinal adult trichinellae. Nine other structurally related naphthoquinones had no such effect.

[2] Bueding, E., and Peters, L., unpublished observations.

[3] Stannard, J. W., McCoy, O. R., and Latchford, W. B., *Am. J. Hyg.*, 1938, **27**, 666.

16791

## Activity of Pantothenol as Pantothenic Acid in Promoting Chick Growth.*

D. MARK HEGSTED. (Introduced by F. J. Stare.)

*From the Department of Nutrition, Harvard School of Public Health, and the Department of Biological Chemistry, Harvard Medical School, Boston.*

The activity of pantothenol as pantothenic acid in various species has been recently reviewed.[1] It is of interest that this compound interferes with the utilization of pantothenic acid by certain microorganisms[2] whereas in rats[3] and human beings[4,5] it is converted into pantothenic acid and appears to be as active as pantothenic acid itself in these two species. From the results presented in this paper, it is concluded that on an equivalent basis, molecule for molecule, pantothenol is 86% as active as pantothenic acid in promoting chick growth.

Nine groups of white leghorn cockerels one week of age were fed the low pantothenic acid diet previously described.[6] After 4 days of depletion, graded doses of calcium pantothenate ranging from 16 to 160 $\mu$g per chick per day were administered to 6 of the groups by pipette placed directly in the crop. The remaining 3 groups received pantothenol in the same manner at 40, 65, and 100 $\mu$g per

* Supported in part by grants-in-aid from the Nutrition Foundation, Inc., New York City, the Milbank Memorial Fund, New York City, and Swift & Company, Chicago, Ill.

[1] Anonymous, *Nutrition Rev.*, 1948, **6**, 272.

[2] Snell, E. E., and Shive, W., *J. Biol. Chem.*, 1945, **158**, 551.

[3] Pfalz, H., *Z. f. Vitaminforsch.*, 1943, **13**, 236.

[4] Burlet, E., *Z. f. Vitaminforsch.*, 1944, **14**, 318.

[5] Rubin, S. H., Cooperman, J. M., Moore, M. E., and Scheiner, J., *J. Nutrition*, 1948, **35**, 499.

[6] Hegsted, D. M., and Lipmann, F., *J. Biol. Chem.*, 1948, **174**, 89.

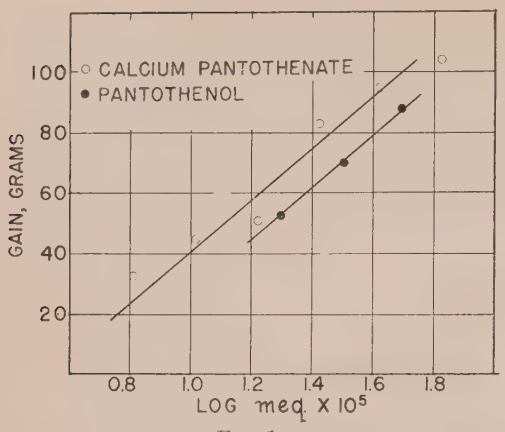



FIG. 1.

A comparison of the gain produced by pantothenic acid and pantothenol in chicks. The equation for the regression line for pantothenic acid is $Y = 86.80X - 59.17$, where Y is gain and X is log dose in milliequivalents $\times 10^5$.

day. The chicks were weighed every other day for 16 days.

The mean gain of each group of 6 chicks is shown in Fig. 1 where the dosage is given as the log of the milliequivalents administered in order to compare the two materials upon a reasonable basis. It is apparent that the log dose-response curve is essentially linear (calculated from the individual gain of each chick) and that the line representing the standard calcium pantothenate is parallel to that for the pantothenol. The potency ratio may therefore be calculated as with other biological assays of this type.[7,8] The distance between the lines in the X direction is 1.932, the antilog of which is 0.857. Thus pantothenol appears to yield 86% of the activity of pantothenic acid when compared upon an equivalent basis. Converting these figures into weights, pantothenol would appear to be about 91% as active as pantothenic acid, and equally as active as calcium pantothenate.

The relative accuracy of this assay compared to other biological assays of this type may be seen by comparing the standard deviation of the dose, s/b, with those tabulated by Bliss and Cattell,[9] where s is the standard deviation about the line and b is the slope. For the calcium pantothenate line these values are 17.14 and 85.18, respectively, and the ratio is 0.201. This value is reasonably small as compared to other vitamin assays.

*Summary.* The chick assay for pantothenic acid, with the supplement administered daily by pipette, yields a reasonably straight log dose-response curve.

Pantothenol was found to have 86% of the activity of pantothenic acid compared milliequivalent for milliequivalent. Gram for gram it was 91% as active.

We are indebted to Merck & Co., Inc., Rahway, N. J., Corn Industries Research Foundation, New York City, and Sheffield Farms Company, Inc., New York City, for generous supplies of materials used in these studies.

The pantothenol was supplied by Hoffmann-La-Roche, Inc., Nutley, N. J.

7 Coward, K. H., The Biological Standardization of the Vitamins, London, Bailliere, Tindall and Cox, 1938.

8 Bliss, C. I., *Ann. App. Biol.*, 1935, **22**, 139.

9 Bliss, C. I., and Cattell, M., *Ann. Rev. Physiol.*, 1943, **5**, 479.

following 50 sites: testis, tunic, cord extension, opposite testicle, cord or tunic, retroperitoneal space, bladder, L. perirenal space, L. kidney, R. perirenal area, R. kidney, R. adrenal, L. adrenal, parietal peritoneum, serosa, omentum and ligaments, intestines, spleen, stomach, liver, pancreas, diaphragm, post-mediastinum, sup. med. and thymus, pleura, lungs, pericardium, heart, ant. cervical region, thyroid, parathyroids, muscle of tongue, muscle of mastication, mandible, teeth (mouth), eyes, pericranium, brain, hypophysis, nose and sinuses, post cerv. region, interscapular space, muscle of scapulae, muscle of thorax and abdo., skin of thorax and abdo., subcut. tissue thorax and abdo., muscle ant. portion of thigh, lower ends of femora, upper ends of tibiae, spinal canal. Paralyzed animals or animals with perforating eye metastases were killed and the day of death listed as the time of occurrence of the paralysis or perforation.

*Results.* Among the 42 New Zealand white rabbits inoculated intratesticularly there were at the end of a 60-day observation period, 37 with primary tumors which had not regressed (88%), 39 with metastases (93%), and 26 which had died from tumor metastases (mortality 62%). The metastatic foci averaged 13.4 (sites) per animal with metastases, 12.3 (sites) per animal with tumor at necropsy, and 11.4 (sites) per animal inoculated (Var. mean = 2.1). The primary tumors averaged by water displacement at necropsy 16.2 cc per animal with primary tumor, 16.2 cc per animal with tumor, and 15.0 cc per animal inoculated. The metastatic tumor averaged 105.6 cc by water displacement at necropsy per animal with metastases, 97.4 cc per animal with tumor and 90.5 cc per animal inoculated.

Among the 66 New Zealand white rabbits inoculated subcutaneously there were at the end of the 60-day observation period 31 with primary tumor which had not regressed (47%), 33 with metastases (50%), and 9 which had died from the effects of tumor metastases (mortality 13%). The metastatic foci averaged 8.2 (sites) per animal with metastases, 6.4 (sites) per animal with tumor, and 3.2 (sites) per animal inoculated (Var. mean = 0.59). The primary tumors averaged by water displacement at necropsy 48.6 cc per animal with primary tumor, 45.7 cc per animal with tumor at necropsy, and 22.8 cc per animal inoculated. The metastatic tumor averaged by water displacement at necropsy 33.2 cc per animal with metastases, 26.1 cc per animal with tumor, and 13.1 cc per animal inoculated.

Each of the above listed differences between the course of tumor after transplantation by the subcutaneous and by the intratesticular routes was statistically significant.[3] For instance, the difference in the mortality of 13% and 62% was significant ($X^2 = 25.1$, $N = 1$, $P = 0.0001$—), and in the number of metastatic foci per animal inoculated of 11.4 and 3.2 (diff. $= 8.2 \pm 1.64$; $t = 5.0$; $P = 0.0001$—).

Generally, the two best criteria for this neoplasm are the mortality from the tumor in

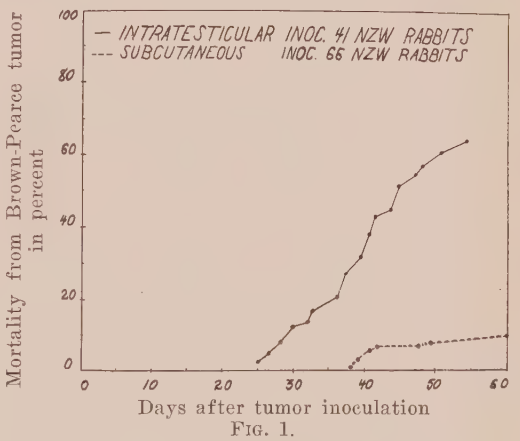
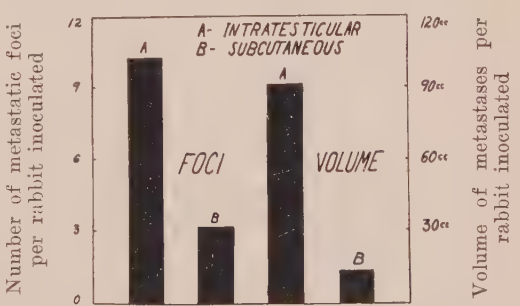



FIG. 1.




Brown Pearce tumor in New Zealand white rabbits

FIG. 2.

[3] Fisher, R. A., Statistical Methods for Research Workers, 1938, 7 ed., Oliver and Boyd, London.

days after inoculation, and the number of metastatic foci determined by careful necropsy and confirmed by microscopic sections.[2] These values are shown in Fig. 1 and 2.

*Discussion.* The total primary and metastatic tumor per animal with metastases was 82 cc per animal inoculated subcutaneously, and 122 cc per animal inoculated intratesticularly; or an average per metastatic focus of 8.9 cc (for the 9.2 primary and metastatic foci) in the former, and 8.4 cc (for the 14.4 primary and metastatic foci) in the latter. It is obvious that the average rate of growth of the tumor was approximately the same in any given site. Under the skin the primary tumor grew larger as there was abundant area to expand. In the testis the growth was confined and spread up the cords to the perirenal areas, retroperitoneal space and peritoneal cavity by simple extension. It has been proven that the pattern of local spread is determined by the site of injection of this tumor;[4] also the pattern of the distant metastases in the Brown-Pearce and in other tumors is not altered or affected by the site origin of the primary tumor[4] nor by the breed of rabbit employed.[5]

The differences between the subcutaneous and the intratesticular routes were not due to the season of the year in which the animals were inoculated.[6] The rabbits inoculated during October, November, December, January, February and March averaged 12.2 metastatic foci among the 16 rabbits transplanted intratesticularly and 4.68 foci among the 22 transplanted subcutaneously; the rabbits inoculated during May, June, July, August and September averaged 10.9 metastatic foci for the 21 rabbits transplanted intratesticularly and 2.48 metastatic foci for the 44 rabbits transplanted subcutaneously. Nor were the differences due to age since the elimination of the 7 animals 3 months of age did not appreciably affect the mean number of metastatic foci or the mortality.

The possibility was considered that the New Zealand Whites obtained from such widely separated areas as Pennsylvania, Illinois, and Alabama might represent stock variations. Such stock variations undoubtedly did occur but the animals injected by the two routes were obtained in roughly proportioned numbers from the various areas. Furthermore, Havana, Himalayana, and Flemish stock obtained also from dealers in the various states have given almost the same data on mortality and metastatic foci as was obtained from inbred lines some 15 years ago.[1] It still remains true that no Havana rabbit has died from the Brown-Pearce tumor, although metastases have occasionally been noted. Approximately 30% of all animals (including all breeds and hybrids) inoculated subcutaneously have died from metastases within a 2-3 months observation period. This may explain a variety of results reported by various authors trying to produce immunity by the subcutaneous route. Nozu was the first to observe metastases of the Brown-Pearce tumor by intracutaneous inoculation alone[7] and Casey induced metastases by the intracutaneous route using the Brown-Pearce XYZ factor.[8]

Brown and Pearce noted the marked tendency to metastasis following intratesticular inoculation of the rabbit tumor and an absence of metastasis following intra- and subcutaneous inoculation.[9] Pearce and Brown were the first to point out that rabbits inoculated with the Brown-Pearce tumor intra- or subcutaneously or intramuscularly "developed an immunity which was sufficient to protect them from subsequent inoculation by the same route or from inoculation made into the

4 Casey, Albert E., and Pearson, Bjarne, PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 234.

5 Casey, Albert E., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 223.

6 Brown, Wade H., Pearce, Louise, and Van Allen, C. M., PROC. SOC. EXP. BIOL. AND MED., 1924, **21**, 371; Pearce, Louise, and Van Allen, C. M., PROC. SOC. EXP. BIOL. AND MED., 1925, **22**, 448; *J. Exp. Med.*, 1927, **45**, 483; Pearce, Louise, and Brown, Wade H., *J. Exp. Med.*, 1927, **45**, 727.

7 Nozu, Y., *Acta dermat.*, 1933, **26**, 66.

8 Casey, Albert E., *Am. J. Cancer*, 1934, **21**, 776.

9 Brown, Wade H., and Pearce, Louise, *J. Exp. Med.*, 1923, **37**, 799.

testicles."[10] Their observations were confirmed by Casey[8] and by Besredka, Mazat, Besnard, Gross, Bardach, Laval,[11] Saphir, Appel, Strauss,[12] Cheever, and Janeway.[13] Besredka and Gross reported that this immunity could be produced only by the tumor cells and that it could not be induced by the intracutaneous inoculation of normal tissues.[11] In Fig. 1 it will be seen that the New Zealand white rabbit shows no evidence of developing at about 40 days an immunity or resistance following intratesticular inoculation as is true for this same breed following subcutaneous inoculation or for common hybrids by intratesticular inoculation as reported by Brown and Pearce[9,10] and by Malluche.[14]

*Summary and conclusions.* Standard values were compiled for the Brown-Pearce tumor in New Zealand white male rabbits, 42 being inoculated intratesticularly and 66 subcutaneously. The breed was highly resistant to subcutaneous inoculation, (mortality, 13.6%; metastatic foci, 3.2; metastatic tumor 13.1 cc per animal inoculated) and susceptible to intratesticular inoculation (mortality 62.7%, metastatic foci 11.4 and metastatic tumor 90.5 cc per animal inoculated). Between 40 and 60 days after intratesticular inoculation the mortality curve continued to rise at about the same rate as between 20 and 40 days. This was in contrast to the immune or resistance reaction which seemed to set in between 40 and 60 days following subcutaneous inoculation of this breed, or following intratesticular inoculation of most other breeds.

[10] Pearce, Louise, and Brown, Wade H., *J. Exp. Med.*, 1923, **37**, 811.

[11] Besredka, A., Mazat, L., and Besnard, P., *Comp. rend. Acad. d. sc.*, 1935, **201**, 170; Gross, L., *Am. J. Cancer*, 1937, **31**, 609; Besredka, A., and Bardack, M., *Comp. rend. Acad. d. sc.*, 1936, **203**, 2193; Besredka, A., *Cancer Bruxelles*, 1935, **12**, 115; Besredka, A., Mazat, L., Laval, P., and Besnard, P., *Ann. Inst. Pasteur*, 1936, **36**, 125; Besredka, A., and Gross, L., *Ann. Inst. Pasteur*, 1936, **57**, 342; 1938, **60**, 5, 465; 1939, **62**, 253.

[12] Saphir, O., and Appel, M., *Am. J. Cancer*, 1940, **38**, 55; Saphir, O., Appel, M., and Strauss, A. A., *Cancer Research*, 1941, **1**, 545.

[13] Cheever, F. S., and Janeway, C. A., *Cancer Research*, 1941, **1**, 23.

[14] Malluche, H., *Beitr. Z. klin. Chir.*, 1938, **167**, 481.

16794

## Selective Blocking of Host Resistance to Malignant Neoplasm (Brown-Pearce Tumor in New Zealand White Rabbits).

ALBERT E. CASEY, LUCILLE MEYERS, AND GEORGE R. DRYSDALE.*

*From the Departments of Pathology, Birmingham Baptist Hospitals, Birmingham, Ala., and Louisiana State University, School of Medicine, New Orleans, La.*

In the preceding paper[1] it was reported that the New Zealand White rabbit is highly susceptible following intratesticular and highly resistant following subcutaneous transplantation of the Brown-Pearce tumor. In the mortality curve an immune or resistance reaction could be detected at about 40-60 days following subcutaneous inoculation but little or none following intratesticular inoculation of the tumor. The present experiments were designed to test the extent to which the XYZ factor[2] might overcome this immune or resistance reaction of the New Zealand white to this tumor following subcutaneous inoculation.

* The work was aided by a grant from the Committee on Growth of the National Research Council, acting for the American Cancer Society.

[1] Casey, Albert E., Meyers, L., and Drysdale, George R., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 576.

[2] Casey, Albert E., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 816; *Am. J. Cancer*, 1934, **21**, 760; 1936, **26**, 276; 1939, **35**, 354.

TABLE I.
Preparation of and Injection Interval for XYZ Factor from Brown-Pearce Tumor (134 New Zealand Whites).

| Exp. | Preparation: Temp. | Preparation: Storage, days | Preparation: Covering | Injection XYZ: Site | Injection XYZ: Dosage, g | Injection XYZ: Interval, days | Animals inoculated Brown-Pearce tumor: Exp. | Animals inoculated Brown-Pearce tumor: Controls |
|---|---|---|---|---|---|---|---|---|
| 1. a. | 24°F | 15 | Paraffin | S.C. | 0.0001 | 19 | 4 S.C. | 4 S.C. |
| b. | 24 " | 15 | " | " | 0.01 | 19 | 5 " | 5 " |
| c. | 24 " | 15 | " | " | 1.0 | 19 | 7 " | 7 " |
| 2. a. | 34 " | 25 | Glycerin | " | 0.0001 | 16 | 2 " | 2 " |
| b. | 34 " | 25 | " | " | 0.01 | 16 | 3 " | 3 " |
| c. | 34 " | 25 | " | " | 1.0 | 16 | 3 " | 5 " |
| 3. | —22°C | *30?* | None | " | 0.02 | 15 | 9 " | 8 " |
| 4. | —22 " | *30?* | " | " | 0.02 | 16 | 5 " | 8 " |
| 5. a. | 0°F | 244 | Glycerin | I.T. | 0.1 | 11 | 3 " } | 4 " |
| b. | 0 " | 244 | " | " | $(0.1)^2$ | 11 | 4 " } | 4 " |
| 6. a. | 0 " | 296 | " | " | 0.1 | 11 | 4 " } | 4 " |
| b | 0 " | 296 | " | " | $(0.1)^2$ | 11 | 4 " } | 4 " |
| 7. | 0 " | 51 | None | S.C. | 0.1 | 21 | 1 " | 2 " |
| | | | | Subtotal (Subcutaneous) | | | 54 | 52 |
| 1. | 18 " | 14 | Paraffin | I.T. | 0.1 | 14 | 5 I.T. | 8 I.T. |
| 2. | 18 " | 10 | " | " | 0.1 | 10 | 6 " | 9 " |
| | | | | Subtotal (Intratesticular) | | | 11 | 17 |
| | | | | Total subcutaneous and intratesticular | | | 65 | 69 |

*Material and Methods.* There were 13 experiments involving 106 New Zealand white rabbits inoculated subcutaneously and several experiments involving 28 New Zealand white rabbits inoculated intratesticularly. In the former 52 controls and 54 experimental animals were inoculated subcutaneously with 0.2 to 0.3 cc of an emulsion of tumor tissue, and in the latter 17 controls (including 6 stock animals) and 11 experimental animals inoculated intratesticularly with a similar dosage.

The XYZ material consisted of aseptically removed Brown-Pearce tumor (from 7 different animals) covered with paraffin, or layered with 50% glycerine in normal saline, or even left in a sterile container uncovered. The tumor tissue was refrigerated at 24°-34° F for 15-25 days (always covered with paraffin or glycerin at these temperatures), or at 0° F or lower for periods up to 300 days (Table I). Immediately after removal from the ice chamber amounts of the refrigerated tumor tissue (in the equivalent of 0.0001 to 1.0 g) were emulsified in normal saline and given as a single injection (see exceptions below) subcutaneously or intratesticularly 11-21 days prior to subcutaneous or intratesticular transplantation of the Brown-Pearce tumor (Table I). One group (in Experiments 5 and 6) was injected twice with XYZ material after a week's interval. The variations in the dosage of the frozen material seemed not to be significant.[3] About half of the animals were obtained directly from the breeder and it was often possible to divide them so that one litter mate would be in the experimental and one in the control group. In any event the controls and experimental animals were matched according to breed and weight before beginning the experiments with equal numbers in each. About 10% of both experimental and control animals died from intercurrent disease, and were eliminated, thus accounting for the unequal groups (Table I). The ex-

[3] Casey, Albert E., *Cancer Research*, 1941, **1**, 134.

TABLE II.

Brown-Pearce Tumor in New Zealand White Rabbits as Influenced by the Prior Injection of the Brown-Pearce XYZ Factor (Subcutaneous Transplantation of Tumor).

| | Non-regressed tumor, necropsy Animals | % | Metastases Animals | % | Died from metastases Animals | % | Total animals |
|---|---|---|---|---|---|---|---|
| XYZ | 39 | (72) | 35 | (65) | 25 | (46) | 54 |
| Controls | 24 | (46) | 17 | (33) | 4 | ( 8) | 52 |
| Chi square | 9.7 | | 10.9 | | 19.9 | | |
| Prob. | 0.01 sig. | | 0.01 sig. | | 0.01 sig. | | |

Metastatic Foci (or Sites) per Animal.

| | Inoculated Mean | Variance | With tumor Mean | Variance | With metastases Mean | Variance |
|---|---|---|---|---|---|---|
| XYZ | 8.0 | 1.6 | 11.1 | 2.3 | 12.4 | 2.4 |
| Controls | 2.6 | 0.6 | 5.6 | 2.4 | 7.9 | 3.7 |
| Diff. | 5.4 ± 1.5 | | 5.5 ± 2.2 | | 4.5 ± 2.5 | |
| Prob. | 0.01 sig. | | 0.01 sig. | | 0.07 | |

Volume of the Metastases per Animal.

| | Inoculated Mean, cc | Variance | With tumor Mean, cc | Variance | With metastases Mean, cc | Variance |
|---|---|---|---|---|---|---|
| XYZ | 38.7 | 55.9 | 53.6 | 88.4 | 59.8 | 99.5 |
| Controls | 11.2 | 25.1 | 24.3 | 106.8 | 34.4 | 195.2 |
| Diff. | 27.5 ± 9.0 cc | | 29.3 ± 13.9 cc | | 25.4 ± 17.1 cc | |
| Prob. | 0.01 sig. | | 0.03 prob. sig. | | 0.15 | |

Volume of Primary and Metastatic Tumor per Animal.

| | Inoculated Mean, cc | Variance | With tumor Mean, cc | Variance |
|---|---|---|---|---|
| XYZ | 60.4 | 135.9 | 83.6 | 214.4 |
| Controls | 17.8 | 53.7 | 38.5 | 223.3 |
| Diff. | 42.6 ± 13.8 cc | | 45.1 ± 20.9 cc | |
| Prob. | 0.01 sig. | | 0.03 prob. sig. | |

TABLE III.

Brown-Pearce Tumor in New Zealand White Rabbits as Influenced by the Prior Injection of the Brown-Pearce XYZ Factor (Intratesticular Transplantation of Tumor).

| | Non-regressed tumor, necropsy XYZ | Controls | Incidence of metastases XYZ | Controls | Died from metastases XYZ | Controls | Total inoculated XYZ | Controls |
|---|---|---|---|---|---|---|---|---|
| Animals | 11 | 16 | 11 | 16 | 11 | 11 | 11 | 17 |
| Percent | (100) | (94) | (100) | (94) | (100) | (65) | (100) | (100) |

| | Metastatic foci (sites) | | Volume of metastases, cc | | Total tumor primary and metastatic, cc | | | |
|---|---|---|---|---|---|---|---|---|
| Per animal inoculated | 23.5 | 13.7 | 170.3 | 53.8 | 189.4 | 73.3 | 11 | 17 |
| | diff. 9.8 ± 3.0 | | diff. 116.5 ± 22.1 | | diff. 116.1 ± 19.5 | | | |
| | t = 3.2 | | t = 5 | | t = 6.0 | | | |
| | P = 0.01—sig. | | P = 0.01—sig. | | P = 0.01—sig. | | | |
| Per animal with tumor | 23.5 | 14.6 | 170.3 | 57.3 | 189.4 | 77.9 | 11 | 16 |
| | diff. 8.9 ± 2.3 | | diff. 113.0 ± 23.6 | | diff. 111.5 ± 25.1 | | | |
| | t = 3.87 | | t = 4.8 | | t = 4.4 | | | |
| | P = 0.01—sig. | | P = 0.01—sig. | | P = 0.01—sig. | | | |

perimental and control animals of two groups were only 3 months of age at the time of inoculation; the others were 4-12 months of age. All except one were males (the exception was among the 54 XYZ animals inoculated subcutaneously).

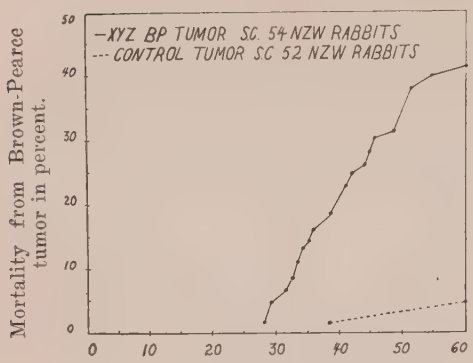
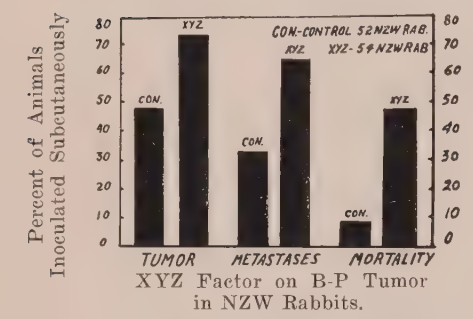
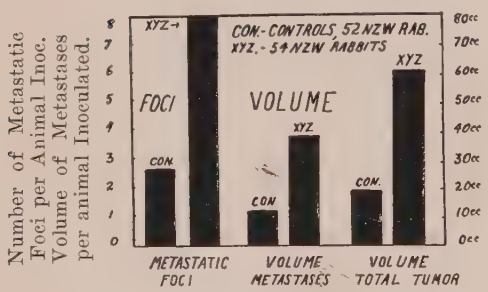



FIG. 1.




XYZ Factor on B-P Tumor in NZW Rabbits.




XYZ Factor on B-P Tumor in NZW Rabbits.

FIG. 2.

Both the control and experimental animals in each group were transplanted with the same emulsion and dosage of Brown-Pearce tumor. The animals were observed at weekly intervals and surviving animals sacrificed at 50 to 90 days. Careful necropsies according to a plan described in the preceding paper, were checked in each instance by the preparation of slides for microscopic examination. The size of the primary and metastatic tumor was measured at necropsy by water displacement.

*Results.* Among the 54 XYZ animals in-

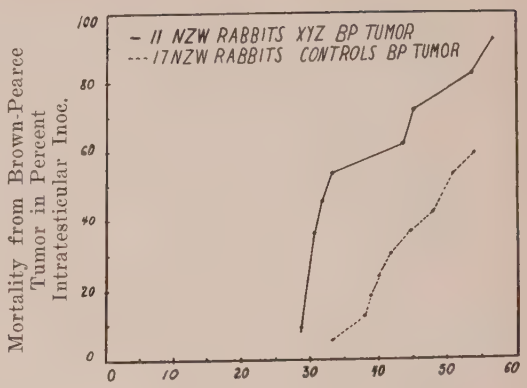



FIG. 3.

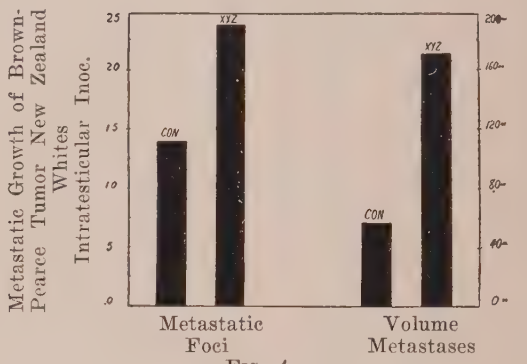



FIG. 4.

oculated subcutaneously and the 11 XYZ animals inoculated intratesticularly with the Brown-Pearce tumor there was a greater incidence of and larger primary tumors, a greater incidence and volume of, and more numerous metastases and a greater mortality from the tumor in a shorter interval after inoculation than among their respective controls (52 controls were inoculated subcutaneously and 17 intratesticularly. Tables II and III, Fig. 1, 2, 3 and 4). Each of the differences was statistically significant for the groups inoculated subcutaneously and for those inoculated intratesticularly with two exceptions. The exceptions relate to the intratesticular inoculation in that 16 of the 17 controls (94%) had non-regressed primary tumor and metastases at necropsy, as compared with 11 (100%) among the XYZ injected animals. This afforded no opportunity to show a statistically significant XYZ effect.

*Discussion.* The production of a state of

hypersusceptibility to tumor transplantation has been, during the past fifty years, fraught with great difficulties. The only recorded instance where published observations could be confirmed by other workers was the demonstration by Haaland,[4] by Leitch,[5] and by Casey[6] that such a state could be produced in the mouse using Bashford carcinoma 63. Sterile Bashford carcinoma 63 tissue was frozen and preserved anaerobically in the frozen state for 10-21 days. An emulsion of the frozen tumor tissue, no longer capable of growth, was injected subcutaneously into mice 10-21 days before subcutaneous transplantation of the same tumor. The animals so injected grew larger primary tumors than their controls not so injected. Some 200 control and 200 experimental animals were employed by the three authors. No metastases were observed and no further observations have been recorded.

Casey[2] was the first to demonstrate that similarly frozen and anaerobically preserved Brown-Pearce tumor tissue, no longer capable of growth, would when injected (1.0 to 0.0001 g) prior to tumor transplantation result not only in a greater incidence of and larger primary tumors but also in a greater incidence. number and volume of the metastases and in a greater mortality from the tumor in a shorter interval after transplantation than in control animals not so injected. The factor responsible was thermolabile[7] probably specific for the Brown-Pearce tumor (homologous),[6,8] filtrable through a Berkefeld "V" filter, differed from the Duran-Reynals spreading factor and effective even when injected into animals carrying the tumor. Because the phenomenon had no known counterpart in biology and the nature of the factor was unknown, the name

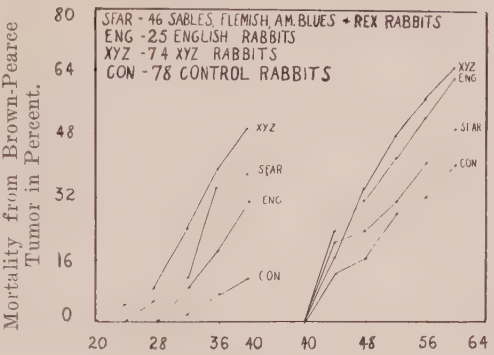


FIG. 5.

"XYZ" was given. Our total experience to date with the unaltered Brown-Pearce XYZ factor (including the present data) consists of 14 experiments involving 74 XYZ and 78 control animals in which the material was injected 10-21 days prior to intratesticular inoculation of this tumor, and 11 experiments involving 84 XYZ and 79 control animals inoculated subcutaneously. In each one of the 25 experiments during a 17 year period was the XYZ effect observed. The mortality curves for the groups inoculated intratesticularly were calculated first for the period up to 40 days and secondly for the period of 40-61 days after tumor transplantation and are presented (Fig. 5). The separation of mortality into the two periods was affected because the host factors influencing the first 40 days seem to differ from the host factors influencing the course of the tumor 40 to 60 days.[10,12] The animal breeds used varied widely but were always matched between controls and experimental animals; the consistency of the results bears this out.

Animals of average resistance to this neo-

4 Haaland, M., *Proc. Roy. Soc. London*, 1910, **82**, 293; *Lancet*, 1910, **1**, 787.

5 Letich, A., *Lancet*, 1910, **1**, 991.

6 Casey, Albert E., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 674.

7 Casey, Albert E., and Moragues-Gonzales, Vincent, *Am. J. Cancer*, 1940, **38**, 59.

8 Casey, Albert E., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 1025; 1934, **31**, 663; 1939, **42**, 731.

9 Casey, Albert E., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 111.

10 Malluche, H., *Beitr. Z. klin. Chir.*, 1938, **167**, 481.

11 Kidd, John G., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 292; *J. Exp. Med.*, 1946, **83**, 227.

12 Casey, Albert E., and Drysdale, George R., *Cancer Research*, 1947, **7**, 728.

13 Casey, Albert E., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 666.

plasm (controls) became somewhat more susceptible than the animals of the most susceptible breeds (English, Flemish, Sable, American Blue and Rex) (Fig. 5). The factor has not been obtained (using identical techniques) from other neoplasms of the mouse or rabbit, nor from the rabbit spleen, skeletal muscle, or rabbit testis. The effect of the material has not in our hands resembled anaphylaxis or sensitization of the usual sort. Necroses do not appear at the site of repeated injections and animals injected weekly for almost a year were still highly susceptible and none died because of the injections. Histologically there seems less necrosis in the tumor tissue of XYZ animals and more cells seem alive. The prolonged storage in the frozen state seems to inactivate or mask such inhibitory factors as may be present in the fresh tumor tissue or the surrounding intercellular fluids.[12]

Kidd,[11] using our technique, froze Brown-Pearce tumor tissue and kept it frozen for 1-2 months at -22° C until ready for use. He then injected a saline emulsion of the frozen and non-viable tumor tissue, in the manner of our experiments, 7-20 days prior to intramuscular transplantation of the tumor. Among the 16 "blue cross" control rabbits was one animal at 30-35 days with progressively enlarging primary tumor (6%), none had metastases (0%) and none died from the tumor (0%). Among the 53 "blue cross" XYZ animals (called "immunized animals" by Kidd) there were 18 at 30-35 days with progressively enlarging primary tumors (34%), at least 10 had metastates (19%) and 10 died from metastases (19%). Evaluation of his work is difficult since his experiments were terminated at 30-35 instead of at the usual 60 days, control animals were few and autopsies were incomplete in that no enumerations of the incidence, number and volume of the metastases was recorded. Nevertheless his "immunized" (XYZ injected) animals had significantly more enlarging tumors ($X^2=4.7$, $P=0.035$), and among those with tumors a greater mortality from metastases ($X^2=5.6$, $n=1$, $P=0.02$) than their respective controls. The term, "immunized," used by Kidd with respect to the XYZ injected animals is, we believe, ill considered.

Several years ago Dr. Kidd kindly sent us 2 samples of frozen Brown-Pearce tumor tissue tested by him for antigen. This nonviable tumor tissue was injected by us into New Zealand white rabbits 15-16 days prior to subcutaneous transplantation of the tumor. The animals comprise Groups 3 and 4 in the present experiments (Table I). Among the 16 controls 3 had non-regressed tumors at necropsy (19%), 2 had metastases (13%), and none had died (0%); the metastatic foci averaged 1.3 and the total tumor 3.6 cc per animal inoculated. Among the 14 XYZ animals 8 had non-regressed tumor at necropsy (57%), 5 had metastases (36%), 4 died from metastases (29%); the metastatic foci averaged 5.8 and the total tumor 26.4 cc per animal inoculated. Combining Dr. Kidd's animals with enlarging primary tumors at 30-35 days with our animals having non-regressed primary tumor at 60 days there were 26 such animals among the 67 "immunized" (XYZ injected), as compared with 4 among the 32 controls ($X^2=7.1$, $n=1$, $P=0.01$– significant). Similarly the deaths from tumor were 14 among the 67 "immunized" (XYZ) and none among the 32 controls ($X^2=8.8$, $n=1$, $P=0.01$– significant).

Kidd like ourselves encountered the XYZ phenomenon while trying to immunize with frozen tumor tissue. An antibody response to the frozen tumor tissue was obtained by him in 22 of the 53 "blue cross" rabbits in the form of complement fixing antibodies with titers of 1-2 to 1-64; and each of the 13 rabbits which failed to grow a primary tumor was in this group. Such an antibody response was absent or insignificant among rabbits of other breeds and mixtures when tested by Cheever,[14] by Jacobs and Houghton[15] and by Kidd.[11] The antibody response seems largely limited to the "blue cross" rabbit, which is a Lilac cross developed by Dr. Wade H. Brown for stock use at the Rocke-

[14] Cheever, F. S., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 517.

[15] Jacobs, J. J., and Houghton, J. D., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 88.

feller Institute. The Lilac and its close relative, the Havana, have the highest natural resistance of any breeds tested against the Brown-Pearce tumor. No Havana rabbit has ever died from the tumor. Dmochowski[16] and Jacobs and Houghton[15] comment that it is "necessary to exclude the obvious likelihood that the antigenic substance is part of the cellular elements of the tumor" which result "in the formation of antibodies" when injected into a genetically different host. The antibodies described by Kidd seem to bear no relation to the growth of the Brown-Pearce tumor in animals successfully transplanted (in direct contrast to the XYZ effect). Among the 9 animals with antibodies and successfully transplanted 5 had enlarging primary tumors at the termination of the experiments (55%) and there were 2 deaths from metastases (22%), as compared with 13 enlarging primary tumors (42%) and 8 deaths from metastases (26%) among the 31 animals without antibodies and successfully transplanted. The differences were not significant.

Known mammalian viruses[17] are thermolabile (55-60°C) yet Kidd has applied the term "virus" to the thermostable (65°C) antigen in the cells of the Brown-Pearce tumor causing antibody response in the Lilac cross or Blue cross rabbit. He disregards the specific filtrable thermolabile (56°C) XYZ factor, uniformly present in the frozen Brown-Pearce tumor tissue, and capable of abrogating or completely blocking host resistance to this neoplasm. Its effect is not limited to certain strains of rabbits. The only biologically comparable filtrable thermolabile factor in mammalian tumors is the milk factor, discovered more recently by Bittner.[18] This agent is also specific, thermolabile (56°C), filtrable, and also does not immunize but renders even resistant strains of mice highly susceptible to the development of a specific neoplasm. It also causes no necrosis, or obvious reaction in the host.

*Summary and Conclusions.* 1. Of 134 New Zealand White rabbits in 15 experiments 65 were given an injection of 1.0 to 0.0001 g of Brown-Pearce tumor tissue (no longer viable) which had been kept frozen anaerobically for 10-296 days. Viable Brown-Pearce tumor tussue was transplanted 10-21 days later into the testes of 54 and beneath the skin in 11 of the 65 animals; also inoculated at the same time were the 69 controls, 52 subcutaneously and 17 intratesticularly.

2. The experimental animals had a significantly greater incidence of and larger primary tumors, a greater incidence and volume of, and more numerous metastases and a greater mortality from the tumor in a shorter interval after inoculation than their respective controls, by both the intratesticular and subcutaneous routes.

3. This effect (XYZ factor) was not confined to the New Zealand White breed as common rabbit hybrids of average resistance to the Brown-Pearce tumor could be rendered more susceptible than even the most susceptible breeds such as the English, Sable, Flemish and Rex. Even the relatively resistant blue-cross or Lilac cross rabbit became more susceptible upon injection of the frozen tumor tissue.

4. The mechanism of the XYZ phenomenon seems to be influenced by the inactivation or masking by prolonged freezing of inhibitory factors present in the fresh tumor. The frozen material seems to act by blocking host resistance to a specific tumor.

16 Domochowski, L., *Compt. rendue de la Soc. de Biol.*, 1938, 349.

17 Seiffert, Gustav, Virus Diseases in Man, Animal and Plant, Philosophical Libr., New York, 1944, 49.

18 Bittner, J. J., *Science*, 1936, **84**, 162; Andervont, H. B., Mammary Tumors in Mice, A.A.A.S., Washington, 1945, 123.

16795

# Effect of Nutrients Upon Growth of Streptomycin-Sensitive, -Resistant- and -Dependent Strains of *Escherichia coli.**†

WARREN P. IVERSON AND SELMAN A. WAKSMAN.

*From the Department of Microbiology, New Jersey Agricultural Experiment Station, Rutgers University.*

Among the various constituents of the medium which influence the antibacterial potency of streptomycin, salt, glucose and certain organic acids were found[1,2] to be of special importance. The presence in nutrient broth of pyruvic and fumaric acids in 1% concentrations enabled *Escherichia coli* to grow in the presence of 10 μg/ml of streptomycin, whereas in the same medium free from these acids, the organism was inhibited even by 1 μg/ml of the antibiotic. With the isolation of streptomycin-dependent strains of this organism, it was considered of importance to determine how the growth of such dependent strains (*E. coli* ds) would be affected by the presence in the medium of those substances which normally favor growth of the original streptomycin-sensitive cultures (*E. coli* ss) in the presence of streptomycin.

*Effect of NaCl upon the growth of E. coli ds.* Varying amounts of NaCl, sterilized by autoclaving, and streptomycin pasteurized by heating for 30 minutes at 60°C, were added to sterile nutrient broth (0.5% peptone and 0.3% meat extract in distilled water) in tubes. These were inoculated with a suspension of *E. coli* ds grown on nutrient agar containing 10 μg/ml streptomycin. Each tube was thus inoculated with 44,000 viable cells. The cultures were incubated at 28°C and the amount of growth was measured by using a Cenco-Sheard-Sanford Photelometer type B2 with a red filter. Growth was expressed as the average turbidity (100–transmittancy) of three tubes.

*E. coli* ds did not grow in the media free from streptomycin; in the presence of streptomycin, however, good growth occurred (Table I). The presence and concentration of NaCl had an important effect. Without the salt, growth was poorer and was more sensitive to higher concentrations of streptomycin, so that 500 μg/ml was sufficient to inhibit completely the growth of the organism. With an increase in concentration of NaCl to 1%, there was a tendency for more rapid initial growth with increasing concentrations of streptomycin. In other words, the presence of NaCl exerts a favorable effect upon the growth of both *E. coli* ss and *E. coli* ds in the presence of streptomycin: The injurious effect of streptomycin upon *E. coli* ss is reduced by the presence of NaCl, and growth of *E. coli* ds is favored.

*Influence of carbon sources upon the growth of* E. coli *ds.* A study was made next of the effect of glucose and of salts of pyruvic and fumaric acids upon the growth of *E. coli* ds (Table II). The above nutrient broth (free from NaCl) was used. When no other carbon source was added, growth was rather limited; an increase in the concentration of streptomycin above 5 μg/ml had a somewhat delaying and even depressing effect upon the growth of the organism. When glucose was added to the broth there was a marked increase in the amount of growth produced; increasing the concentration of streptomycin above 5 μg/ml had again a delaying effect upon the rate of growth. Pyruvate and fumarate also exerted a highly favorable effect upon the growth of *E. coli* ds. This was especially true of the pyruvate; in this case, however, an increase in the concentration of the streptomycin above

---

* Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

† Partly supported by a grant made by the Commonwealth Fund of New York.

1 Green, S. R., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 281.

2 Green, S. R., Iverson, W. P., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 285.

TABLE I.
Influence of NaCl on Growth of *E. coli* ds.

| | No NaCl | | | | | 0.4% NaCl | | | | | 1.0% NaCl | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Concentration of streptomycin, μg/ml | | | | | | | | | | | | | | |
| Hrs of incubation | 0 | 5 | 25 | 100 | 500 | 0 | 5 | 25 | 100 | 500 | 0 | 5 | 25 | 100 | 500 |
| | Turbidity. | | | | | | | | | | | | | | |
| 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 19 | 0 | 7 | 0 | 0 | 0 | 0 | 11 | 10 | 4 | 0 | 0 | 0 | 10 | 7 | 1 |
| 25 | 0 | 13 | 2 | 0 | 0 | 0 | 15 | 14 | 13 | 0 | 0 | 1 | 14 | 19 | 12 |
| 43 | 0 | 18 | 13 | 0 | 0 | 0 | 18 | 19 | 19 | 5 | 0 | 11 | 20 | 19 | 16 |
| 91 | 0 | 18 | 16 | 9 | 0 | 0 | 26 | 24 | 24 | 20 | 0 | 14 | 28 | 26 | 24 |
| 139 | 0 | 15 | 13 | 12 | 0 | 0 | 25 | 22 | 21 | 16 | 0 | 17 | 21 | 19 | 18 |

TABLE II.
Influence of Carbon Sources upon the Growth of *E. coli* ds.

| | No added carbon source | | | | Glucose | | | | Pyruvate | | | | Fumarate | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Concentration of streptomycin, μg/ml | | | | | | | | | | | | | | | |
| Hrs of incubation | 0 | 5 | 15 | 25 | 0 | 5 | 15 | 25 | 0 | 5 | 15 | 25 | 0 | 5 | 15 | 25 |
| | Turbidity. | | | | | | | | | | | | | | | |
| 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 17 | 0 | 1 | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 2 |
| 24 | 0 | 10 | 4 | 0 | 0 | 23 | 14 | 5 | 0 | 2 | 19 | 23 | 0 | 1 | 12 | 17 |
| 41 | 0 | 18 | 18 | 13 | 0 | 36 | 38 | 35 | 0 | 19 | 38 | 42 | 0 | 11 | 26 | 25 |
| 95 | 0 | 21 | 22 | 18 | 0 | 44 | 45 | 43 | 0 | 27 | 50 | 52 | 0 | 22 | 38 | 40 |
| 119 | 0 | 20 | 21 | 16 | 0 | 48 | 49 | 46 | 0 | 33 | 52 | 53 | 0 | 23 | 40 | 43 |

TABLE III.
Influence of Carbon Sources in a Synthetic Medium upon the Growth of *E. coli* ss in Presence of Streptomycin.

| | Glucose | | Pyruvate | | Fumarate | |
|---|---|---|---|---|---|---|
| | Concentration of streptomycin, μg/ml | | | | | |
| Incubation, hr | 0 | 5 | 0 | 5 | 0 | 5 |
| | | | Turbidity | | | |
| 24 | 0 | 0 | 0 | 0 | 0 | 0 |
| 48 | 0 | 0 | 10 | 0 | 4 | 0 |
| 72 | 2 | 0 | 14 | 0 | 9 | 0 |
| 96 | 21 | 0 | 11 | 0 | 9 | 0 |
| 120 | 25 | 0 | 14 | 0 | 13 | 0 |
| 142 | 26 | 0 | 16 | 0 | 15 | 0 |
| 169 | 30 | 0 | 20 | 0 | 18 | 0 |
| 193 | 32 | 0 | 21 | 0 | 23 | 0 |
| 265 | 35 | 0 | 27 | 0 | 28 | 0 |
| 336 | 36 | 0 | 30 | 0 | 33 | 0 |
| 528 | 39 | 0 | 43 | 0 | 42 | 0 |

5 μg/ml not only had no delaying effect but proved to be more favorable to growth. These results thus tend to prove that pyruvate and fumarate favor the growth of both *E. coli* ss and *E. coli* ds, in the presence of streptomycin.

*Use of synthetic media for studying the effect of carbon sources upon streptomycin activity on various E. coli strains.* In order to eliminate the interfering action of peptone and meat extract upon the effect of supplementary addition of carbon sources, a synthetic (Koser's) medium was used. This medium consisted of 1 g $(NH_4)H_2PO_4$, 1 g $K_2HPO_4$, 0.2 mg $MgSO_4 \cdot 7H_2O$, 5 g NaCl, 1000 ml distilled water, and was adjusted to pH 7.3. The carbon sources, previously sterilized by passage through a Seitz filter, were added to make a final concentration of 0.2%. Several strains of *E. coli* of varying sensitivity to streptomycin were used in these

TABLE IV.

Influence of Carbon Sources in a Synthetic Medium upon the Growth of *E. coli* ds in Presence of Streptomycin.

| | Glucose | | | | Pyruvate | | | | Fumarate | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Concentration of streptomycin, μg/ml | | | | | | | | | | | |
| Incubation,hr | 5 | 50 | 500 | 1000 | 5 | 50 | 500 | 1000 | 5 | 50 | 500 | 1000 |
| | Turbidity | | | | | | | | | | | |
| 24 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 49 | 0 | 0 | 0 | 0 | 1 | 1* | 0 | 0 | 0 | 0 | 0 | 0 |
| 66 | 0 | 0 | 0 | 0 | 4 | 5 | 0 | 0 | 0 | 2† | 4 | 1† |
| 90 | 0 | 0 | 0 | 0 | 13 | 13 | 0 | 0 | 1* | 5 | 9 | 5 |
| 118 | 0 | 0 | 0 | 0 | 16 | 15 | 10 | 0 | 3 | 10 | 15 | 14 |
| 138 | 0 | 0 | 1* | 0 | 18 | 16 | 8 | 0 | 5 | 13 | 19 | 18 |
| 164 | 0 | 13 | 10 | 0 | 19 | 18 | 17 | 4† | 7 | 16 | 22 | 22 |
| 193 | 2* | 23 | 20 | 0 | 22 | 20 | 19 | 16† | 10 | 20 | 23 | 23 |
| 261 | 23* | 28 | 22 | 21* | 24 | 22 | 21 | 10* | 14 | 27 | 23 | 21 |
| 384 | 24 | 32 | 26 | 23 | 26 | 25 | 24 | 21 | 22 | 26 | 16 | 15 |
| 648 | 30 | 36 | 30 | 30 | 38 | 37 | 18 | 15 | 32 | 30 | 16 | 15 |

Measurements reported are averages of 3 tubes, except those indicated by * where growth occurred in 2 tubes only and † with growth in one tube only. No growth in media free from streptomycin.

TABLE V.

Influence of Carbon Sources in a Synthetic Medium upon the Growth of *E. coli* rs‡ in Presence of Streptomycin.

| | Glucose | | | | | Pyruvate | | | | | Fumarate | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Concentration of streptomycin, μg/ml | | | | | | | | | | | | | | |
| Incubation, hr | 0 | 5 | 50 | 500 | 1000 | 0 | 5 | 50 | 500 | 1000 | 0 | 5 | 50 | 500 | 1000 |
| | Turbidity | | | | | | | | | | | | | | |
| 31 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 46 | 0 | 0 | 0 | 0 | 0 | 0 | 1* | 1 | 0 | 0 | 1† | 1* | 1* | 0 | 0 |
| 71 | 0 | 0 | 0 | 0 | 0 | 11 | 16 | 16 | 4 | 0 | 5 | 7 | 8 | 7 | 2* |
| 96 | 0 | 0 | 0 | 0 | 0 | 19 | 18 | 20 | 16 | 0 | 10 | 11 | 12 | 13 | 8 |
| 120 | 0 | 0 | 0 | 0 | 0 | 22 | 20 | 23 | 18 | 7 | 15 | 14 | 16 | 17 | 16 |
| 144 | 13† | 0 | 0 | 0 | 0 | 24 | 22 | 26 | 23 | 17 | 18 | 18 | 22 | 22 | 21 |
| 168 | 16 | 9* | 6† | 0 | 0 | 28 | 24 | 27 | 25 | 21 | 21 | 21 | 24 | 24 | 23 |
| 240 | 24 | 25 | 22 | 0 | 26† | 33 | 29 | 32 | 29 | 26 | 27 | 27 | 32 | 28 | 24 |
| 336 | 28 | 31 | 27 | 26† | 24 | 36 | 32 | 34 | 30 | 26 | 29 | 25 | 25 | 19 | 15 |
| 528 | 31 | 35 | 31 | 34† | 31† | 44 | 40 | 41 | 26 | 16 | 34 | 30 | 26 | 19 | 16 |

‡ This strain was obtained from a culture of *E. coli* ss growing in presence of streptomycin.

experiments, namely the streptomycin-sensitive (ss), streptomycin-resistant (rs) and streptomycin-dependent (ds).

Growth of *E. coli* ss was inhibited by 5 μg/ml of streptomycin, as shown in Table III. The presence of pyruvate and fumarate in the medium did not overcome the inhibiting effect of the antibiotic in the above concentration. *E. coli* ds grew well in all tubes containing streptomycin, varying in concentration between 5 to 1000 μg/ml (Table IV). With the higher amounts of streptomycin, growth with glucose was somewhat better than with pyruvate and fumarate. As one would expect, no growth occurred in the media free from streptomycin.

A streptomycin-resistant strain of *E. coli* (rs) was obtained by allowing the parent culture to grow in nutrient broth containing 1 μg/ml of streptomycin, plating out the culture at the end of a month's incubation, using agar media containing 1000 μg/ml streptomycin, and isolating one of the colonies. This culture of *E. coli* rs grew best with pyruvate as a source of carbon, both in the absence and in the presence of streptomycin (Table V). An increase in concentration of the antibiotic resulted in a delaying effect upon growth of the culture in the presence of glucose, and to a lesser extent in the presence of the salts of the organic acids.

A second resistant strain was obtained by plating out a 28 hour old nutrient broth culture of the parent sensitive strain of *E. coli*

TABLE VI.
Growth of *E. coli* rs* in a Synthetic Medium Containing Different Carbon Sources and Various Concentrations of Streptomycin.

| | Glucose | | | Pyruvate | | | Fumarate | | |
|---|---|---|---|---|---|---|---|---|---|
| | Concentration of streptomycin, μg/ml | | | | | | | | |
| Incubation, hr | 0 | 5 | 500 | 0 | 5 | 500 | 0 | 5 | 500 |
| | Turbidity | | | | | | | | |
| 98 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 122 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| 146 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8 |
| 168 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 13 |
| 192 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 19 |
| 213 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 19 |
| 238 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 20 |
| 265 | 0 | 0 | 0 | 0 | 0 | 15 | 0 | 0 | 21 |
| 290 | 0 | 0 | 0 | 0 | 0 | 15 | 0 | 0 | 20 |
| 362 | 0 | 0 | 0 | 0 | 0 | 15 | 0 | 0 | 15 |
| 432 | 0 | 0 | 0 | 0 | 0 | 22 | 0 | 0 | 13 |
| 624 | 0 | 0 | 0 | 0 | 0 | 16 | 0 | 0 | 13 |

* This strain was isolated from the plating of *E. coli* ss in agar containing streptomycin (15 μg/ml).

using nutrient agar to which 15 μg/streptomycin had been added. Of the colonies which appeared on the plate, one was found to be resistant to streptomycin. Of the remaining ten, 5 were similar to the first in resistance to streptomycin (in other words, they were *E. coli* rs strains) and 5 were dependent on streptomycin (*E. coli* ds strains). The first colony gave rise to a culture which grew only in synthetic media in the presence of pyruvate or fumarate as a carbon source and with streptomycin only in concentrations of 500 μg/ml (Table VI); no growth took place in higher (1000 μg/ml) or lower (100 μg/ml) concentrations. When such growth in the pyruvate or fumarate tubes was streaked on plain nutrient agar or on agar containing 10 μg/ml of streptomycin, all grew on the streptomycin agar but not on the streptomycin-free agar. Two of the 3 pyruvate tubes also gave some growth on the plain agar. When this freshly isolated *E. coli* ds was inoculated into nutrient broth, allowed to grow for 4 days at 28°C, then plated out on plain agar and on streptomycin-containing (10 μg/ml) agar, 1 ml of the culture contained 137,000,000 sensitive cells and 470,000 dependent cells of *E. coli*. A streptomycin-resistant strain was thus transformed, by growing in a fumarate or pyruvate medium, first into a streptomycin-dependent and then into a streptomycin-sensitive strain.

A preliminary experiment in which various amino acids were added to the synthetic medium with glucose as a carbon source seemed to indicate that l-lysine was necessary for the growth of this resistant organism.

These results thus bring out emphatically the relationship between the nutrition of *E. coli* and its sensitivity or resistance to or dependence on streptomycin.

*Summary.* 1. Growth of a streptomycin-dependent strain of *E. coli* in nutrient broth was favored by the presence of sodium chloride.

2. Glucose, pyruvate, and fumarate, when added to nutrient broth minus sodium chloride, caused greater growth of the streptomycin-dependent strain. With nutrient broth alone and nutrient broth plus glucose, the greatest initial growth took place in the presence of 5 μg streptomycin/ml. Nutrient broth plus the sodium salts of pyruvate and fumarate, however, favored a greater initial growth in higher concentrations of streptomycin (25 μg/ml).

3. In a synthetic medium containing glucose, pyruvate and fumarate as carbon sources, the growth of the sensitive parent *E. coli* strain was inhibited by 5 μg/ml; the dependent strain grew in all concentrations of streptomycin from 5 to 1000 μg/ml, but not in media without streptomycin. One resistant strain grew in all concentrations of

streptomycin as well as in media without streptomycin, while a second resistant strain grew only in a streptomycin concentration of 500 μg/ml with pyruvate and fumarate as carbon sources.

4. This second streptomycin resistant strain was transformed into a streptomycin-dependent strain under the above conditions. The dependent strain so obtained remained dependent after several transfers on nutrient agar containing streptomycin, and in turn gave rise to streptomycin-sensitive cells when a large inoculum was placed in streptomycin-free nutrient broth.

16796 P

## A Simple Technic for Counting Megakaryocytes.*

EDWIN E. OSGOOD.

*From the Division of Experimental Medicine, University of Oregon Medical School.*

*Principle of method.* Make a thick drop preparation of an accurately measured volume of marrow as in examination for malaria and count all the megakaryocytes in the preparation.

*Method.* From a well-shaken, evenly suspended marrow specimen,[1] fill a Sahli hemoglobin pipette to the 20 cmm mark and blow contents onto a clean slide to form a thick drop. Spread evenly with the pipette tip over an oval area about 1.5 × 3 cm. Usually several such thick drop slides are made so that additional slides are available for examination in case part of the drop is detached by too vigorous washing and so that, when the count is low, a statistically significant number of cells can be counted.[1] Place slides in an incubator overnight. The following morning, immerse the slides in a solution of 5% formalin in 1% acetic acid in a Coplin jar until the erythrocytes are lysed and the thick drop is grayish in color. Flood the laking solution off the slide with water and gently rinse with buffer-phosphate solution (pH 6.4).[2] Stain with Wright's stain alone for 2 to 3 minutes. Add buffer-phosphate solution and stain 60 to 90 minutes. Wash slide by holding horizontally under gently running water. Air dry. Spread a thin film of immersion oil over the smear and examine systematically, using a mechanical stage and 200 × magnification, preferably with an 8 mm objective which is not immersed in the oil. Count all megakaryocytes seen in the specimen and multiply by 50 to express as number per ml. If the total nucleated marrow cell count per cmm and the leukocytic-erythrocytic ratio are determined, the number of megakaryocytes per million nucleated marrow cells or per million nucleated erythrocytic cells may be calculated. Morphology is fairly well preserved and any cell can be examined in greater detail by switching to the oil immersion lens.

In previously reported methods a count was made of all megakaryocytes and all nucleated marrow cells[3] or of all megakaryocytes and all nucleated erythrocytic cells[4] in an area of arbitrarily determined size on a thin marrow smear and results expressed, respectively, as megakaryocytes per million nucleated marrow cells or per million nucleated erythrocytic cells. These methods have the disadvantages that they are extremely time-consuming and that such large cells are seldom distributed evenly and, indeed, are likely to be found in greatest numbers at the tails of the smear. The area selected for examination will thus

* Aided by a grant from the George A. Myers Research Fund for Study of Hemorrhagic Diseases.

1 Osgood, E. E., and Seaman, A. J., *Physiol. Rev.*, 1944, **24**, 46.

2 Osgood, E. E., "Laboratory Diagnosis," p. 478, 3rd ed., Blakiston.

3 Limarzi, L. R., and Schleicher, E. M., *J.A.M.A.*, 1940, **114**, 12.

4 Dameshek, W., and Miller, E. B., *Blood*, 1946, **1**, 27.

greatly influence the count. If results are expressed as number per million nucleated erythrocytic cells, megakaryocytes are actually more numerous than results would indicate in diseases characterized by erythrocytic hyperplasia such as blood loss anemia due to purpura hemorrhagica and actually less numerous than the results would indicate in erythrocytic hypoplasia. The same objections apply when the result is expressed as number per million nucleated marrow cells and, in addition, leukocytosis, leukemia, and other hyper- or hypoplastic diseases of the leukocytic system would distort the apparent number of megakaryocytes. For these reasons, it would be preferable to determine the absolute number per ml.

Study by this thick drop technic of the number of megakaryocytes found in normal and pathologic marrows is under way, but not yet complete. Preliminary results would seem to indicate that the normal range is between 500 and 4000 megakaryocytes per ml; between 20 and 150 per million nucleated marrow cells; and between 50 and 300 per million nucleated erythrocytic cells.

16797

## Influence of Amino-Acids on Adrenal Enlargement, Nephrosclerosis and Hypertension by Anterior Pituitary Preparations.*

OLGA B. HENRIQUES,† S. B. HENRIQUES,† R. DEGRANDPRÉ, AND H. SELYE.

*From the Institut de Médecine et de Chirurgie Expérimentales, Université de Montréal.*

Previous investigations carried out in this laboratory have shown that overdosage with lyophilized anterior pituitary (LAP) can produce nephrosclerosis and hypertension in rats fed a 30% casein diet.[1] It was also observed that different kinds of proteins[2] and protein hydrolysates[3] are as effective as casein in the production of nephrosclerosis and hypertension by LAP. This paper is concerned with the nephrosclerotic activity of LAP in animals fed a diet in which the protein component was provided by a mixture of crystalline amino-acids.

*Material and methods.* 25 hooded black-and-white male rats weighing 110 to 130 g were divided into 4 groups. Groups I and II received a "22%" amino-acid‡ diet and Groups III and IV a "22%" Amigen‡ (enzymatic casein hydrolysate) diet, both rations containing 4% sodium chloride. In order to maintain equal food intake, all animals received their diet as a 70 g % aqueous suspension, 3 times daily at 6-hour intervals through a stomach tube. First Groups I and II were adapted to this procedure by forced-feeding mixtures of Amigen and amino-acids in which the proportion of Amigen was gradually decreased so that on the 10th day only amino-acids were fed. At the same time, Groups III and IV were adapted to forced feeding with the Amigen diet. The volume and caloric value of the food given was the same in all groups. The amount of food was so adjusted that the animals slightly increased in weight.

After the adaptation period, all animals

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service, and by the Commonwealth Fund.

† From the Laboratory of Endocrinology, Instituto Butantan, São Paulo, Brazil.

1 Dontigny, P., Hay, E. C., Prado, J. L., and Selye, H., *Am. J. Med. Sciences*, 1948, **215**, 442.

2 Hay, E. C., Prado, J. L., and Selye, H., *Canad. J. Research* (Section E, Med. Sc.), 1948, **26**, 212.

3 DeGrandpré, R., Prado, J. L., Dontigny, P., Leduc, J., and Selye, H., *Fed. Proc.*, 1948, **7**, 27.

‡ "22%" means a nitrogen content corresponding to 22% casein. For composition of diets see Tables I and II.

TABLE I.
Composition of Amino-acids Mixture.

| | g |
|---|---|
| *l*-(+)-arginine hydrochloride | 5.8 |
| *l*-(+)-histidine " | 11.9 |
| *l*-(+)-lysine " | 30.0 |
| *dl*-isoleucine | 24.0 |
| *l*-(—)leucine | 21.6 |
| *dl*-methionine | 14.4 |
| *dl*-phenylalanine | 16.8 |
| *dl*-threonine | 28.8 |
| *dl*-tryptophane | 4.8 |
| *dl*-valine | 33.6 |
| *l*-(+)-glutamic acid | 137 |
| *l*-(—)-tyrosine | 20.0 |

TABLE II.
Composition of Diets (Parts %).

| | Amigen | Amino-acids diet |
|---|---|---|
| Amigen | 30* | — |
| Amino-acids mixture | — | 37* |
| Corn starch | 28 | 28 |
| Sugar | 30 | 30 |
| Cod liver oil | 1 | 1 |
| Fat | 1 | 1 |
| Steenbock's mineral mixture | 4 | 4 |
| Sodium chloride | 4 | 2.5 |
| Cellu flour | 1 | 1 |
| Charcoal | 5 | 5 |
| Vitamins[4] | 10 ml | 10 ml |
| Sodium bicarbonate† | — | 21.3 |

* These amounts correspond in nitrogen to 22 g of casein.

† Used for neutralization of the amino-acids hydrochlorides.

were castrated and unilaterally nephrectomized. LAP treatment was started the following day in Groups I and III, and continued for 27 days. LAP was administered in 2 daily subcutaneous injections of 0.1 cc of 20 mg/day during 15 days, this dose being raised to 30 mg for 6 days and to 40 mg during the last 6 days.

The blood pressures were determined on the 27th day of treatment by the direct method, as previously described,[1] the animals being killed next day.

The severity of nephrosclerosis was designated in terms of a scale of 0 to +++. The criteria employed microscopically were arteriolonecrosis, capillary tuft hyalinization and cast formation with resultant tubule dilatation and atrophy.

*Discussion.* As may be seen from Table III, adrenal enlargement, nephrosclerosis and hypertension can be produced by LAP, on a

TABLE III.
Effect of Amino-acids Content of the Diet on Hypertension Produced by LAP.*

| Groups | No. rats | Treatment | Body wt | | Nephrosclerosis‡ | | Adrenal wt, mg | Kidney wt, mg/100 cm² b. surface | Blood pressure, mm Hg. |
|---|---|---|---|---|---|---|---|---|---|
| | | | Initial | Final | Incidence, % | Severity, % | | | |
| 1 | 6 | "22%"† amino-acids + LAP | 128 | 189 ± 3.5 | 50 | 22 | 72 ± 6.1 | 663 ± 51 | 143.8 ± 20 |
| 2 | 6 | "22%" amino-acids | 126 | 169 ± 2.2 | 0 | 0 | 32 ± 1.2 | 420 ± 18 | 115.5 ± 13.9 |
| 3 | 6 | "22%" Amigen + LAP | 126 | 225 ± 3.0 | 100 | 75 | 92 ± 11 | 629 ± 18 | 140.6 ± 6.3 |
| 4 | 7 | "22%" Amigen | 124 | 194 ± 2.2 | 0 | 0 | 39 ± 1.3 | 324 ± 11.3 | 129.4 ± 10.8 |

* Lyophilized anterior pituitary.
† "22%" means a nitrogen content corresponding to 22% casein.
‡ Nephrosclerosis as observed by microscopic examination.

diet in which amino-acids completely replace proteins. This finding indicates that the constituent amino-acids are responsible for the conditioning, by dietary proteins, to the above toxic effects of LAP overdosage. Our data also show that the growth, adrenal enlargement and nephrosclerosis produced by LAP were less pronounced on the amino-acid diet than on the Amigen diet of equal nitrogen content. It may incidentally be mentioned that the usual myocarditic changes[5] which accompany this type of hypertension were also less obvious in the amino-acid than in the Amigen groups. This could possibly be explained by the fact that the animals of Groups I and II received an unnatural mixture of amino-acids. Yet the existing difference between Groups I and III is of sufficient magnitude to suggest that equal amounts of amino-N are not necessarily equally conducive to the production of nephrosclerosis by LAP.

*Summary.* Adrenal enlargement, nephrosclerosis and hypertension were produced by a lyophylized anterior pituitary (LAP) preparation in rats kept on a synthetic diet containing no protein, but an adequate amount of amino-acids. The severity of the lesions was less marked, however, than in rats receiving an equivalent amount of nitrogen in the form of a casein-hydrolysate. It appears that amino-acids suffice to sensitize the organism to the production of hypertensive disease by LAP. This is noteworthy since no such pathologic changes can be produced by LAP on other diets deficient in proteins.

[4] Hawk, P. B., Oser, B. L., and Summerson, W. H., *Practical Physiological Chemistry*, The Blakiston Co., Philadelphia, 1947.

[5] Selye, H., *J. Clin. Endocrinol.*, 1946, **6**, 117.

16798

## Comparative Assays of Rodenticides on Wild Norway Rats. II. Acceptance.*

SALLY H. DIEKE. (Introduced by C. P. Richter.)

*From the Psychobiological Laboratory, Phipps Psychiatric Clinic, Johns Hopkins Hospital, Baltimore, Md.*

In the previous paper of this series,[1] 8 common rodenticides were compared on the basis of their acute toxicities to recently trapped wild Norway rats. As was pointed out in that paper, a high toxicity does not, however, guarantee effectiveness under field conditions; of at least equal importance are those properties, such as a bitter taste or the lack of it, which influence the voluntary consumption of poisoned baits. It is the purpose of the present work to reexamine the same 8 rodenticides in the light of their acceptability and effectiveness when offered to wild Norway rats in a standard bait under controlled conditions in the laboratory.

*Methods.* The 565 wild Norway rats on which data are here given were trapped in the city of Baltimore during the period from July 1946 to December 1947. Before use they were kept in the laboratory, in large holding cages, for a few days; this delay proved necessary to allow for occasional deaths or for signs of debility to become evident. They were maintained on Purina fox chow and water during this time. The healthy specimens were then transferred (in almost every instance within 2 weeks after trapping) to individual wire mesh cages provided with a metal food cup and a water bottle. The food cup had fitted over it an aluminum

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

[1] Dieke, S. H., and Richter, C. P., *Pub. Health Rep.*, 1946, **61**, 672.

cover with a small oval opening, designed to minimize spillage, and it rested directly under a matching hole cut in one corner of the wire mesh bottom of the living compartment.

The bait used was a special yellow corn (Funks' Hybrid G-94, grown in McLean County, Illinois†), freshly ground to a uniform fineness for each week's work. The cornmeal was weighed into the food cups and placed in the cages at 4:30 P.M. for the rats to eat overnight. Next morning at 9:30 a.m. the food cups were removed and reweighed. Extra cups containing cornmeal were occasionally weighed in the afternoon and again next morning to make sure that atmospheric conditions did not cause changes in weight which might reduce the accuracy of the observations. No changes of more than 0.2 grams ever occurred, which was considered within the experimental error.

The cups were marked and always returned to the same cage. The rats were purposely left without food during the day in order to prevent them from starting to eat before dusk, which is their normal feeding time under field conditions.

After 2 nights of prebaiting with plain corn, according to the above schedule, poison was added to the cornmeal for the third night. To ensure a uniformly small particle size, each sample was first ground in a mortar for 2 or 3 minutes; it was then added to the appropriate amount of cornmeal and blended in a mechanical mixer for 15 to 20 minutes. The poisoned baits were always freshly prepared on the day the rats received them.

Next morning the poisoned food was removed and weighed, and any rats alive at this time were fed fox chow until they died or were discarded (4 to 10 days after poisoning, depending on the poison and the condition of the rat). A large number of rats were autopsied, including all those not dying promptly, in order to exclude data on rats not succumbing to the poison alone. No surviving rat was ever used again.

Most of the experiments were performed during the autumn, winter and spring months. No seasonal variation in toxicity was found in previous work, and since the present work likewise gave no indication of any, the data have been considered as a whole. A difference in response attributable to sex was previously found to exist for none of the poisons except red squill; so a separation of data on male and female rats is here made only for that poison. And although data on young rats are included in the table giving nightly consumptions of plain corn, no sexually immature rats were used in the poison experiments, thus obviating any variation attributable to age.

The poisons used came from the same containers as in the preceding work, with the exception of sodium fluoroacetate (1080) and arsenic trioxide, supplies of which had been exhausted. The new samples of these two poisons were reassayed by stomach tube, according to the method previously described,[1] and 52 additional wild Norway rats were used to check the toxicity figures previously obtained for the other poisons. No substantial changes in toxicity were detected and accordingly the LD50 figures previously obtained for strychnine sulfate, alpha-naphthyl thiourea (ANTU), thallium sulfate, fortified red squill and barium carbonate were allowed to stand.

*Results. Plain Cornmeal.* The average amounts of unpoisoned cornmeal eaten by the rats during the prebait period are shown in Table I. They have been broken down according to size of rat and sex, to show that the average consumption increased from 6 to 10 g for immature rats up to more than 18 g for full grown males. Individual large rats had intakes as high as 30 g on the second night, and a few ate more than 25 g both nights.

In each weight range the females seem to have eaten somewhat less than the males, although not significantly less in the young adult classes (100 to 299 g body weight). On the average all but the small rats ate more the second night than they did the first, indicating a certain hesitation to eat what was presumably an unfamiliar food. Only 24 rats (not included in the tabulation) refused

† Provided through the courtesy of Funk Brothers Seed Company, Bloomington, Ill.

TABLE I.
Consumption of Cornmeal on 2 Successive Nights by 565 Wild Norway Rats.*

| Wt range | No. rats | Avg body wt, g | Avg consumption, g | |
|---|---|---|---|---|
| | | | 1st night | 2nd night |
| Below 50 g | 4 ♂ | 47.0 | 8.0 | 7.1 |
| | 3 ♀ | 47.0 | 6.3 | 6.0 |
| 50- 99 | 49 ♂ | 80.0 | 10.3 | 10.0 |
| | 63 ♀ | 73.8 | 9.7 | 9.4 |
| 100-199 | 51 ♂ | 140.5 | 10.7 | 11.1 |
| | 86 ♀ | 144.5 | 10.3 | 11.2 |
| 200-299 | 74 ♂ | 249.0 | 12.7 | 14.6 |
| | 77 ♀ | 251.5 | 13.5 | 14.5 |
| 300-399 | 63 ♂ | 342.1 | 15.6 | 17.8 |
| | 60 ♀ | 343.4 | 13.9 | 16.4 |
| 400-499 | 19 ♂ | 435.5 | 16.4 | 18.4 |
| | 13 ♀ | 427.4 | 14.3 | 16.4 |
| >500 | 3 ♂ | 543.0 | 11.8 | 17.0 |
| | 0 ♀ | — | — | — |

* Not including data on 23 additional rats which refused to eat the first night, and 1 rat which refused both nights.

to eat any cornmeal on the first night, a ratio of approximately one refusing rat in 24, or 4%, and of these all but one ate well the second night.

*Poisons.* The kills obtained with various percentages of each poison are given in Table II, which also lists for comparison the acute median lethal doses (LD50's) of the poisons as determined by stomach tube administration. The lowest poison concentrations at which complete kills were obtained are seen to range from 0.1% for thallium sulfate to 5% for female rats receiving squill. No bait containing strychnine sulfate or barium carbonate killed all the rats receiving it, nor was any concentration of red squill completely successful against both male and female rats.

In general, the higher the concentration of any poison, the more rats died from eating it. This was, however, not true for strychnine sulfate and squill: for these, some fatalities occurred throughout the range but increasing the concentration did not increase the efficiency of the bait.

The reduced consumptions of bait resulting from the addition of the various poisons to the cornmeal are shown in Table III, which gives data on the same rats as Table II. This shows that the amount of bait consumed decreased fairly regularly with increasing content of poison. At the levels marked with asterisks, which were the lowest concentrations killing all the rats used, the average intakes were reduced to 14% of prebait for 1080, 21% for female rats receiving squill, 28% for ANTU, and 30% for zinc phosphide. No reduction at all followed the introduction of thallium sulfate into the bait at the equivalently lethal concentration. Reduced intakes are seen also for strychnine and barium carbonate, but even with these poisons a total refusal to eat never occurred.

*Discussion.* From a practical standpoint, experiments such as these serve only to indicate what will happen under field conditions. Just as toxicity figures based on administration by stomach tube represent the irreducible minimum of poison which is likely to kill, so results with trapped and individually caged wild Norways merely show what percentages of poison in bait can be expected to kill under the most favorable conditions in the field. Captive wild rats have been removed from their normal environment and may well be suspicious of any food offered them; on the other hand they are not subject to distrac-

TABLE II.
Comparative Killing Concentrations.
(Voluntary consumption of poisons in cornmeal bait by wild Norway rats; mortality ratios (rats dying/rats used) for each poison are listed opposite the appropriate concentrations. The median lethal doses and their standard errors are added for comparison at the bottom of the table.)

| Conc. in bait | Strychnine 1080 (36 rats) | Strychnine $SO_4$ (20 rats) | ANTU (36 rats) | $Tl_2SO_4$ (36 rats) | $Zn_3P_2$ (40 rats) | $As_2O_3$ (24 rats) | Squill (24 ♀, 24 ♂) | | $BaCO_3$ (20 rats) |
|---|---|---|---|---|---|---|---|---|---|
| .01% | 2/ 4 | | | | | | | | |
| .02 | 1/ 4 | | 0/4 | 0/ 4 | | | | | |
| .05 | 10/12 | 1/4 | 4/8 | 11/12 | 2/8 | | | | |
| .1 | 7/ 8 | 0/4 | 1/4 | 8/ 8 | 1/4 | | | | |
| .2 | 8/ 8 | 5/8 | 5/8 | | 8/8 | | | | |
| .5 | | 0/4 | 4/4 | 8/ 8 | | 0/4 | | | |
| 1 | | | 8/8 | 4/ 4 | 4/4 | 1/4 | | | |
| 2 | | | | | 7/8 | 6/8 | 5/8 | 1/8 | |
| 4 | | | | | | 4/4 | | | |
| 5 | | | | | 8/8 | | 4/4 | 1/4 | |
| 6 | | | | | | 4/4 | | | |
| 10 | | | | | | | 2/4 | 0/4 | 0/4 |
| 15 | | | | | | | | | 3/4 |
| 20 | | | | | | | 2/4 | 0/4 | 3/4 |
| 50 | | | | | | | 2/4 | 2/4 | 7/8 |
| Lowest conc. killing all rats, % | 0.2 | — | 0.5 | 0.1 | 0.2 | 4 | 5 | — | — |
| $LD_{50}$ Acute,* mg/kg | 0.44 | 4.8 | 6.9 | 15.8 | 40.5 | 57.5 | 133 | 276 | 1480 |
| (± S.E.) | ±0.19 | ±0.4 | ±0.5 | ±0.9 | ±2.9 | ±7.0 | ±10 | ±29 | ±340 |

* Values for 1080 and $As_2O_3$ from Table IV, the rest from reference [1].

TABLE III.
Average Voluntary Intakes of Poisoned Cornmeal Showing Lowered Consumptions with Increasing Concentrations of Poisons.*

| Conc. in bait, % | Strychnine 1080 g | Strychnine $SO_4$ g | ANTU g | $Tl_2SO_4$ g | $Zn_3P_2$ g | $As_2O_3$ g | Squill ♀, g | Squill ♂, g | $BaCO_3$ g |
|---|---|---|---|---|---|---|---|---|---|
| 0 (prebait) | 15.4 | 16.0 | 14.5 | 15.2 | 13.8 | 15.6 | 16.4 | 17.4 | 14.7 |
| .01 | 9.1 | | | | | | | | |
| .02 | 2.6 | | 9.0 | 11.0 | | | | | |
| .05 | 3.3 | 7.7 | 8.0 | 15.7 | 10.3 | | | | |
| .1 | 2.4 | 6.1 | 5.7 | 15.5* | 6.4 | | | | |
| .2 | 2.2* | 4.1 | 2.7 | | 4.1* | | | | |
| .5 | | 2.8 | 4.1* | 8.4 | | 2.9 | | | |
| 1 | | | 1.2 | 6.9 | 2.5 | 0.9 | | | |
| 2 | | | | | 1.7 | 1.7 | 9.7 | 8.7 | |
| 4 | | | | | | 1.4* | | | |
| 5 | | | | | 1.3 | | 3.4* | 4.9 | |
| 6 | | | | | | 0.6 | | | |
| 10 | | | | | | | 0.4 | 3.5 | 1.5 |
| 15 | | | | | | | | | 4.0 |
| 20 | | | | | | | 1.0 | 0.2 | 2.4 |
| 50 | | | | | | | 0.8 | 1.3 | 3.4 |

* The prebait figure in each case is the average intake of all rats used for that poison. The intakes of poisoned bait at the lowest concentrations giving complete kills are marked with asterisks. (Mortality data on these same rats are given in Table II.)

tions such as other food supplies, or disturbances resulting from attacks by other rats or natural enemies, which could influence their food consumption. It is, for instance, unlikely that every unwanted rat would have the opportunity or even the inclination to eat 15 or 20 g of a 0.1% thallium sulfate bait at one sitting, and therefore poisoning with so low a concentration, while very efficient in the laboratory, might easily prove futile for

TABLE IV.
Reassays of 1080 and Arsenic Trioxide.
(Administered by stomach tube, in water containing 10% acacia, to recently trapped wild Norway rats (methods described in Ref. 1).

| Poison and source | Dose, mg/kg BW | Mortality | $LD_{50} \pm$ S.E. mg/kg BW |
|---|---|---|---|
| 1080 | 0.2 | 1/4 | $0.44 \pm 0.19$ |
| (Monsanto Chemical Co.) | 0.3 | 2/4 | |
| | 0.5 | 4/7 | |
| | 1 | 4/6 | |
| | 2 | 7/8 | |
| $As_2O_3$ | 25 | 0/4 | $57.5 \pm 7.0$ |
| (Mallinkrodt, analytical) | 50 | 3/8 | |
| | 75 | 3/4 | |
| | 100 | 1/1 | |

practical purposes. But it can be said that any poison not effective in laboratory feeding tests such as these will undoubtedly kill far fewer rats in the field.

On the basis of the above results, then, 1080, ANTU, thallium sulfate, and zinc phosphide should be good and efficient rodenticides; arsenic trioxide (of good grade and with small particle size) slightly less so; while perfect control of Norway rat populations should not be obtainable through the use of red squill, barium carbonate, or strychnine sulfate.

The value of prebaiting is indicated by the fact that rats did not entirely refuse to eat baits containing the very bitter strychnine sulfate, or even concentrations of red squill as high as 50%, after they had had access to the same (unpoisoned) bait for 2 nights previously.

The reduced intakes of bait after adding poison may be ascribed either to early detection of the poison by the rats and subsequent refusal, or to the rapid onset of toxic reactions making the rats too ill to do more than sample the bait. In the case of 1080, which rats do not taste,[2] the second is probably the determining factor. For strychnine sulfate, the bitter taste is undoubtedly the deterrent. For most of the others it is more difficult to distinguish between the two possibilities. Thallium sulfate appears to be entirely undetected by the rats, and, were it not for the hazard to humans involved in its use, would seem to be the ideal rat poison.

*Summary.* The relative efficiencies of 8 common rodenticides were determined by offering the poisons to recently trapped wild Norway rats in a standard bait (freshly ground cornmeal) under standard conditions. The voluntary consumption of baits containing sodium fluoroacetate (1080), alpha-naphthyl thiourea (ANTU), thallium sulfate and zinc phosphide resulted in complete kills at concentrations of 0.5% or lower. A sample of arsenic trioxide of relatively high toxicity required a concentration of 4% for a complete kill. All the female rats offered 5% of a good grade of fortified red squill died, but higher concentrations, and all concentrations offered to male rats, gave only partial kills. Barium carbonate gave good but not complete kills at concentrations up to 50%, while strychnine sulfate killed few rats in the range tested.

The amounts of unpoisoned cornmeal consumed on 2 successive nights, by male and female wild Norway rats in various weight groups were determined. The addition of every poison except thallium sulfate to the cornmeal caused a considerable reduction in intake, owing either to recognition of the poison or to a rapid onset of toxic effects.

2 Richter, C. P., in press.

16799

# Decreases in Plasma Volume During Electrically Induced Convulsions in Man.

M. D. Altschule and J. E. Cline.

*From the Laboratory of Clinical Physiology, McLean Hospital, Waverley, and the Department of Medicine, Harvard Medical School, Boston, Mass.*

Electrically induced convulsions as used in the treatment of mental disease consist of a brief period of generalized muscular contractions of maximal vigor; they are associated with marked rises in intrathoracic and intra-abdominal pressures and the development of anoxia. Studies made here on the effects of electroshock therapy have afforded data bearing on problems connected with the physiology of violent physical exercise; it was considered of interest to describe the changes in plasma volume which occur during this type of muscular exertion.

*Material and methods.* Thirteen subjects were studied, 8 of whom were women. Cardiovascular function was normal except for mild hypertension in several. Two of the subjects were studied twice each, the rest being observed in one treatment each. Three of the women (Cases 11, 12, 13) were studied during convulsions moderated as a consequence of the previous intravenous injection of curare (solution Intocostrin or solution d-tubocurarine Squibb); in one of these (Case 11) observations had also been made during a seizure induced without the previous injection of curare.

The plasma volume was measured by the short indirect method of Gibson and Evans,[1] 4 samples of venous blood being taken over a period of 30 to 40 minutes immediately before the induction of the seizure, and another being taken immediately at the end of the convulsion; in 5 instances an additional sample was taken 15 minutes later. The concentration of dye in each sample of plasma was estimated by means of a Coleman Junior Spectrophotometer. The plasma protein concentration was estimated in 5 patients by means of the copper-proteinate method;[2] measurements of plasma protein so made were used to estimate changes in plasma volume using the following formula:

$$\text{plasma volume after seizure} = \frac{\text{plasma volume before} \times \text{plasma protein before}}{\text{plasma protein after}}$$

*Observations.* The plasma volume was diminished at the end of the convulsive seizure in every instance; the decreases ranged between 50 cc and 1040 cc, or 1.7% to 34.7% of the original values (Table I, Fig. 1). In the case of the men, the decreases were between 9.0% and 34.7% of the control levels, the average being 19.4%. The largest decrease occurred in a young muscular athlete in good condition (Case 1), while the smallest decrease in this group was observed in a weak, malnourished, middle-aged individual who had led a sedentary life for several decades (Case 5). One patient, studied twice, showed diminutions in plasma volume of 25.8 and 17.9% on the two occasions. In the uncurarized women, diminution in plasma volume immediately after the convulsion amounted to between 7.3 and 13.7% of the control values; the average was 10.5%. The curarized women showed decreases of 1.7 to 4.7% of the control values for plasma volume, changes which are not significant; the average change was 3.2%. One woman studied twice showed a decrease in plasma volume of 7.3% when not given curare, and of 3.1% when curarized.

Fifteen minutes after the seizure the plasma volume as a rule was partially restored to its original value (Table I).

*Discussion.* Kaltreider and Meneely[3] re-

[1] Gibson, J. G., II, and Evans, W. A., Jr., *J. Clin. Invest.*, 1937, **16**, 301.

[2] Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Jr., Hamilton, P. B., and Archibald, R. M., *Bull. U. S. Army Med. Dept.*, 1943, **71**, 66.

TABLE I.
Changes in Plasma Volume and Plasma Protein Concentration After Convulsions.

| Case | Sex | Plasma volume | | | | Plasma protein, g % | | |
|---|---|---|---|---|---|---|---|---|
| | | Before, cc | After, cc | Difference, % | 15 min. later, cc | Before | After | 15 min. later |
| 1 | M | 3000 | 1960 | 34.7 | 2740 | 6.86 | 8.17 | 7.53 |
| 2a | M | 3000 | 2225 | 25.8 | | | | |
| 2b | M | 2600 | 2135 | 17.9 | | | | |
| 3 | M | 3130 | 2610 | 16.6 | 2900 | 6.69 | 7.29 | 7.08 |
| 4 | M | 3910 | 3430 | 12.3 | 3430 | 5.45 | 6.44 | 6.08 |
| 5 | M | 3050 | 2775 | 9.0 | | | | |
| | Avg—Men | | | 19.4 | | | | |
| 6 | F | 1420 | 1225 | 13.7 | | | | |
| 7 | F | 1750 | 1520 | 13.1 | | | | |
| 8 | F | 2460 | 2190 | 11.0 | | | | |
| 9 | F | 2140 | 1940 | 9.4 | 2210 | 6.27 | 7.16 | 6.49 |
| 10 | F | 2360 | 2160 | 8.5 | | | | |
| 11a | F | 2120 | 1965 | 7.3 | | | | |
| | Avg—Women | | | 10.5 | | | | |
| | Avg—Men and women | | | 14.9 | | | | |
| 11b | F | 2130 | 2065 | 3.1 | | | | |
| 12 | F | 1910 | 1820 | 4.7 | | | | |
| 13 | F | 3010 | 2960 | 1.7 | 2910 | 5.37 | 5.64 | 5.73 |
| | Avg—Curarized women | | | 3.2 | | | | |

viewed the earlier work on the effect of exercise on the plasma volume and added additional data; much of the previous work was fragmentary or carried out by means of methods of doubtful validity. Indeed, the validity of the method of Gibson and Evans[1] used by Kaltreider and Meneely,[3] and also in the present study, has not been established in exercising subjects. In view of the fact that the dye used in that method is attached to albumin,[4] it is clear that if this protein left the vascular bed during exercise the decreases in plasma volume as measured would be smaller than the actual decreases. It appears, therefore, that the decreases in plasma volume which occur during exertion actually are as large as those reported by Kaltreider and Meneely[3] and in the present study, or possibly greater. A criticism of the validity of the method during exertion advanced by Ebert and Stead[5] is more serious; those authors reported a change in optical quality of the serum after exercise, which in 5 of their 6 experiments was large enough to introduce serious errors into the measurement of plasma volume with the dye method. These errors were detected by Ebert and Stead[5] by means of the large discrepancies found when de-

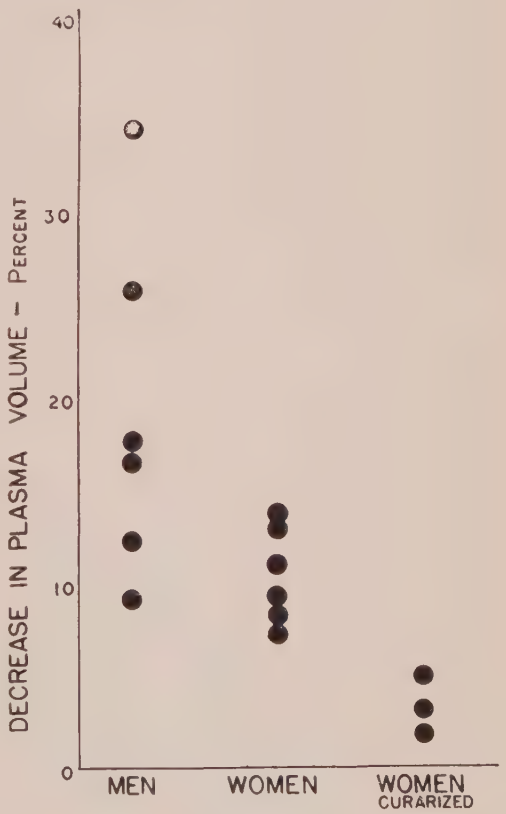



FIG. 1.

[3] Kaltreider, N. L., and Meneely, G. R., *J. Clin. Invest.*, 1940, **19**, 627.

[4] Gregerson, M. I., and Gibson, J. G., *Am. J. Physiol.*, 1937, **120**, 494.

[5] Ebert, R. E., and Stead, E. A., Jr., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 139.

TABLE II.
Plasma Volume Immediately After Convulsion.

| Case | Measured by dye method, cc | Calculated from changes in plasma protein, cc | Avg deviation, % |
|---|---|---|---|
| 1 | 1960 | 2480 | ± 11.7 |
| 3 | 2610 | 2870 | ± 4.8 |
| 4 | 3430 | 3310 | ± 1.8 |
| 9 | 1940 | 1870 | ± 1.8 |
| 13 | 2960 | 2870 | ± 1.5 |

TABLE III.
Plasma Volume 15 Minutes After Convulsion.

| Case | Measured by dye method, cc | Calculated from changes in plasma protein, cc | Avg. deviation, % |
|---|---|---|---|
| 1 | 2740 | 2740 | ± 0 |
| 3 | 2900 | 3060 | ± 2.7 |
| 4 | 3430 | 3510 | ± 1.2 |
| 9 | 2210 | 2050 | ± 3.8 |
| 13 | 2910 | 2820 | ± 1.6 |

creases in plasma volume during exercise were, on the one hand, measured by the dye method, and on the other hand, calculated from changes in plasma protein concentration. In the present study, a similar comparison was made in 10 experiments. In 4 of the 5 based on studies made immediately after the convulsion there was satisfactory agreement between the two methods, *i.e.*, the plasma volumes calculated by the two methods agreed with each other within 4.8% of the mean of each pair of values; there was no consistent directional difference between the two (Table II). In one of these 5 experiments agreement was not as good, the two values differing from each other by 11.7% of their mean. In a comparison of data obtained 15 minutes after the convulsion in 5 experiments, good agreement was found between plasma volumes as measured by the dye method and those calculated from changes in plasma protein level (Table III). It is concluded, therefore, that the criticism of the dye method in exercise made by Ebert and Stead[5] is not substantiated by the present data in that whatever changes in optical properties of serum may occur are not significant in measurements of plasma volume by this method; possibly the longer duration of their exercise or the fact that our subjects were fasting may explain the differences found.

The finding in the present study of decreases in plasma volume in uncurarized subjects of 7.3 to 34.7%, averaging 14.9%, is smaller than expected on the basis of comparison with the data of Kaltreider and Meneely.[3] However, conditions in the experiments of these authors were so different from those of the present study as to invalidate direct comparisons. The rises in venous pressure, and presumably also in capillary pressure, were far greater in our experiments than in those of Kaltreider and Meneely;[3] the increases which occur in uncurarized subjects during electrically induced convulsions average 53 cm of water and in curarized subjects average 23 cm of water.[6] However, as Landis[7,8] pointed out, increased filtering pressure accounts for only a small part of the loss of fluid from the circulation during muscular exercise. Moreover, during electrically induced convulsions the rises in venous and capillary pressures which occur do not become increased effective filtering pressures everywhere, because of the simultaneous, approximately equal, rises in intrathoracic,[9] intra-abdominal[9] and cerebrospinal fluid pressures.[10] Accordingly, blood drawn after the end of a seizure, as in the present study, is a mixture of some blood exposed to high filtering pressures and some exposed to normal filtering pressures in the small vessels. Accordingly, little significance can be attached to the fact that the average decrease in plasma volume found here in uncurarized subjects, *i.e.*, 14.9%, is close to that found by Landis *et al.*[11] *i.e.*, 12.0%, when those authors artificially raised venous pressures in the arm to a level comparable to that reached in patients

[6] Altschule, M. D., and Tillotson, K. J., *Arch. Neurol. and Psychiat.*, in press.

[7] Landis, E. M., *Physiol. Rev.*, 1934, **14**, 404.

[8] Landis, E. M., *Ann. New York Acad. Sci.*, 1946, **46**, 713.

[9] Gordh, T., and Silfverskiold, B. P., *Act. Med. Scandinav.*, 1943, **113**, 183.

[10] Marsan, C. A., and Fuortes, M. G. F., *Riv. di Neurol.*, 1947, **17**, 1.

[11] Landis, E. M., Jonas, L., Angevine, M., and Erb, W., *J. Clin. Invest.*, 1932, **11**, 717.

experiencing induced seizures; the similarity of the findings does not constitute an argument for the importance of increases in filtering pressure in causing the changes in plasma volume observed in the present study.

The effects of other factors which operate during induced seizures are similarly impossible to evaluate. These factors include severe anoxia and hypercarbia,[12] muscular hyperemia with consequent increases in filtering surface due to vigorous muscular contractions[13] and increased transudation of fluid, of uncharacterized etiology, from the vessels of exercising limbs.[8] Likewise, it is impossible to establish significance of the osmotic effects of muscle cell metabolites, considered to result in the drawing of water from the small blood vessels during exercise.[7,8] Until the factors which regulate loss of water from the circulation during exercise can be defined precisely, it will not be possible to analyze the mechanisms responsible for the loss of circulating fluid which occurs during the course of electrically induced seizures. Nevertheless, the data of the present study show a parallelism between the violence of the convulsions induced and the decreases in plasma volume which occurred; the average decrease in men was 19.4%, in uncurarized women 10.5% and in curarized women 3.2%.

It is clear from the present study that the losses of fluid from the circulation, and their possible consequences, are not essential for obtaining a satisfactory clinical result in patients given electroshock therapy.

*Summary and conclusions.* Measurements of plasma volume were made 15 times in 13 subjects in whom convulsions were induced electrically for the treatment of mental disease. Decreases in plasma volume were appreciable and varied in amount with the severity of the seizures induced. The previous administration of curare moderated the convulsions and minimized decreases in plasma volume. It is concluded that the method of Gibson and Evans[1] for measuring plasma volume is valid under the conditions of the present study. It is also concluded that the transitory decreases in plasma volume which occur during electrically induced convulsions are a side reaction and are not essential for obtaining a satisfactory clinical result.

[12] Altschule, M. D., Sulzbach, W. M., and Tillotson, K. J., *Am. J. Psychiat.*, 1947, **103**, 680.

[13] Landis, E. M., *Am. J. Physiol.*, 1931, **98**, 704.

## 16800 P

## Urinary Penicillin Excretion in Women.

MILTON J. GOLDEN, FRANZ M. NEUMEIER, AND KURT A. OSTER.
*From the Research Laboratories, McKesson and Robbins, Inc., Bridgeport, Conn.*

The effectiveness of antacids in preventing penicillin destruction by gastric acidity has been measured by studies of the urinary recovery of orally administered penicillin.[1,2] In these investigations, male subjects had to be studied, since reproducible values were not obtainable with female test persons. This report is concerned with the determination of a possible relationship between renal clearance of penicillin and the menstrual cycle.

*Methods.* Three healthy, young, adult women volunteered for these studies. For each test 25,000 units of commercial sodium penicillin were dissolved in 200 cc of an aqueous solution or suspension of 2 g of buffer or antacid and were taken orally 2 hours after a light breakfast consisting of fruit juice, coffee, and a slice of toast. No food was taken in the following 2 hours. The antacids were the same as previously used:[1] sodium

[1] Golden, M. J., and Neumeier, F. M., *Science*, 1946, **104**, 102.

[2] Perlstein, D., Kluener, R. G., and Liebmann, A. J., *Science*, 1945, **102**, 66.

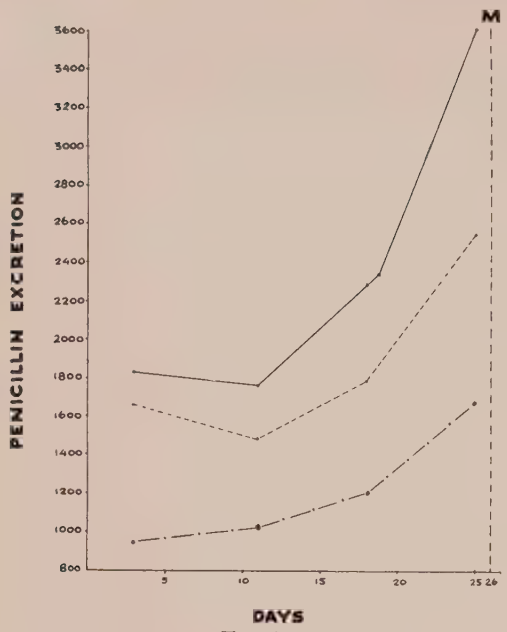



FIG. 1.
Urinary excretion of penicillin in Subject A at various intervals of the menstrual cycle. M signifies the onset of menstruation lasting three days.
———— Sodium bicarbonate
——— • ——— Sodium citrate
– – – – – – – – Antacid mixture

bicarbonate U.S.P., sodium citrate U.S.P. and an antacid powder containing sodium bicarbonate, colloidal kaolin, magnesium trisilicate and bismuth subcarbonate. Physiological saline served as control. Urine was collected in sterile glass-stoppered bottles one-half hour after penicillin administration and thereafter at hourly intervals for 6 hours. The volumes of urine were determined, and the samples were assayed, using the FDA cylinder-plate method of January 1945 (*Staphylococcus aureus* FDA No. 209). A standard penicillin assay curve was prepared daily for comparison with the unknowns.

When the frequency and duration of the menstrual periods were established for each subject, identical doses of penicillin plus antacid were given with reasonable spacing from 4 to 6 times during the cycle, excluding actual menstruation. The test was then interrupted for at least one month to avoid any interference with subsequent doses and possible tolerance. A different antacid was then employed in the following month's tests.

*Results.* Fig 1 is a representative example of the 3 cases studied. It shows penicillin (expressed in Oxford units) recovered from the urine in 6 hours with different buffers at various intervals of the menstrual cycle, plotted against the number of days elapsed since the last day of menstruation. The first one-half hour sample includes the urine already present in the bladder prior to the test. Each point of the graph represents the average of at least 2 determinations.

It is apparent from the data that the recovery of penicillin in the urine is increased just before menstruation. Oral administration of penicillin-saline solutions resulted in small penicillin recovery in the urine, confirming the statement that unbuffered penicillin is absorbed in the intestinal tract to a slight extent.[3]

*Discussion.* The increase in urinary penicillin excretion preceding menstruation may be attributed either to an increased absorption or decreased destruction of penicillin from the gastrointestinal tract, or to an increased secretion. The actual mechanism will be investigated further. The data here presented may aid in explaining the discrepant reports on the urinary penicillin recovery in women after the oral administration of buffered penicillin, carried out without regard to the menstrual cycle.[1,2]

*Summary.* A marked increase in urinary excretion of orally ingested penicillin has been observed in women immediately preceding menstruation. The physiological mechanism of this phenomenon is under investigation.

[3] Rammelkamp, C., and Keefer, C. J., *J. Clin. Invest.*, 1943, **22**, 425.

16801

# Action of Furacin in Delaying Growth of a Transplanted Fibrosarcoma in Mice.*

MORRIS N. GREEN† AND CHARLES E. FRIEDGOOD.‡ (Introduced by Stuart Mudd.)

*From the Departments of Bacteriology and Surgical Research, Medical School, University of Pennsylvania, Philadelphia.*

Relatively little is known regarding the chemotherapy of tumors. Greenberg and Schulman[1] recently suggested that the search for new chemotherapeutic agents against neoplasms might be facilitated by using the "metabolite antagonism" approach. This concept has been extensively applied in research directed towards finding new antibacterial agents.

Furacin§ (5-nitro-2-furaldehyde semicarbazone) inhibits the growth of a large number of gram positive and gram negative bacteria.[2] Green[3] recently has shown that furacin inhibits bacterial enzymes involved in glucose and pyruvate metabolism. The following experiments were designed to test the effect of furacin on growth of a transplanted fibrosarcoma.

*Materials and methods.* Forty adult inbred C3H mice of the Andervont strain were used. They were all transplanted with an equal amount of mouse sarcoma S-13‖ into the right axillary space.

Furacin was prepared for injection by suspending finely powdered crystals in peanut oil, using a concentration of 200 mg per ml. The furacin was administered by subcutaneous injection in the dorsal region.

The animals were divided into 4 groups of 10 each and treated as follows:

*Group I* One week after transplantation, 0.1 ml of furacin suspension (containing 20 mg of furacin) was injected.

*Group II* Three days prior to transplantation, the animals were given 0.1 ml of the furacin suspension followed by another injection of the same dose a week after transplantation.

*Group III* This group were also injected with 0.1 ml of the furacin suspension 3 days prior to transplantation, again after one week and 2 weeks after transplantation.

*Group IV* This was the control group. The tumor was transplanted as in the other groups but no furacin was given.

All the animals were housed in wire cages and were fed Purina Fox Chow stock diet. The tumors were palpated periodically and the progress of growth recorded. At the time of death the animals were autopsied and sections of the tumor, lung, kidney and adrenal glands were prepared for microscopic examination.

*Results.* Table I shows the effect of furacin in prolonging the life span of the tumor-bearing mice. It will be noted in Table I that there was less than one chance in a thousand that the differences in the average survival of the furacin-treated and control groups could be due to chance alone. Although furacin exhibited a definite inhibitory effect on the growth of the tumor, eventually all the animals died as a result of the malignancy. The most effective retardation of growth was obtained with three doses of furacin (Group III). Under these conditions, the

* This work was supported by a grant from the Eaton Laboratories, Inc., Norwich, N.Y.

† Present address: School of Medicine, University of Missouri, Columbia, Mo.

‡ Present address: Mt. Sinai Hospital, New York, N.Y.

1 Greenberg, D. M., and Schulman, M. P., *Science*, 1947, **106**, 271.

§ Furacin was supplied by Dr. L. Eugene Daily of the Eaton Laboratories.

2 Dodd, M. C., *J. Pharm. Exp. Therap.*, 1946, **86**, 311; Shipley, E. R., and Dodd, M. C., *Surg., Gyn., Obst.*, 1947, **84**, 366.

3 Green, M. N., *Fed. Proc.*, 1948, **7**, 305; in press, *Arch. of Biochem.*

‖ The original tumor was supplied by Dr. Margaret R. Lewis of the Wistar Institute. It gives 100% takes on transplantation in this strain of mice.

TABLE I.
Survival Time in Days of Mice After Implantation of S-13 Tumor and Treatment with Furacin.

| Group | Treatment* | Avg survival (M) | Range | Stand. Dev. ($\sigma$) | t† | P‡ |
|---|---|---|---|---|---|---|
| I | 20 mg furacin inj. | 29.2 | 22-34 | 3.55 | 5.76 | >0.001 |
| II | 40 " " | 34.2 | 30-42 | 3.60 | 8.93 | >0.001 |
| III | 60 " " | 37.4 | 30-47 | 4.68 | 9.27 | >0.001 |
| IV | Control | 20.9 | 18-25 | 2.63 | — | — |

* For details of treatment see under description of methods in text. Each group contained ten mice.

† $t = \dfrac{M_1 - M_2}{\sqrt{\frac{\sigma_1^2}{9} + \frac{\sigma_2^2}{9}}}$

‡ A value of $t = 4.781$ gives a chance variation (P) of 0.001.

tumor transplant was not palpable for 2 weeks, while in the control group, the tumor was palpable at the end of 5 days following transplantation. There was no evidence of metastasis in any of the animals; the fibrosarcoma growing locally was well encapsulated. In the treated animals, there was microscopic evidence of cellular degeneration with pyknotic nuclei and decreased mitotic activity of the tumors in the treated mice as distinguished from those in the untreated controls. The furacin was largely absorbed because only negligible amounts of the drug were observed at autopsy at the site of injection.

It is of interest to note that adrenal enlargement was observed in the furacin treated animals. Histologically the fascicular zone of the cortex appeared hypertrophied. The effect of furacin on the adrenal cortex will be reported in a subsequent paper. Using furacin in the doses described above, there was no evidence of parenchymal damage to the liver, kidney or lungs.

*Discussion.* These experiments indicate that furacin may inhibit the growth of neoplastic tissue. The histological findings in the treated tumors indicate that furacin has a selective effect on the tumor tissue. Experiments are being continued to determine which factors explain the action of furacin in inhibiting tumor growth.

*Summary.* Furacin inhibits the growth of mouse sarcoma S-13 in C3H mice. The average survival time of the control mice after implantation of the tumor was 21 days. The average survival time of the furacin-treated mice varied from 29 to 37 days, depending on the amount of the furacin given. Histological examination of the treated tumors reveal some cellular degeneration and a lessened mitotic activity. Hypertrophy of the adrenal cortex and symptoms of B complex vitamin deficiency were observed in the furacin-treated animals.

The authors wish to thank Dr. Margaret Reed Lewis of the Wistar Institute for her generous advice and interest in this problem as well as for cooperation in supplying the tumors and animals used in these experiments. We are also indebted to Dr. Stuart Mudd for his interest and support.

16802

# Effect of "Neurotripsy" on the Partially Denervated Muscle of the Dog.*

J. N. Frederick and A. J. Kosman. (Introduced by J. S. Gray.)

*From the Department of Physiology, Northwestern University Medical School, Chicago, Ill.*

Regenerating nerve fibers may produce an increased number of axone branches. Billig, van Harreveld, and Wiersma[1] have applied this principle in their treatment of paretic muscles by a procedure which they have called "neurotripsy." This technic consists of either crushing the nerve as it enters the paretic muscle—surgical "neurotripsy"—or of a forceful massage of the affected part which is directed at breaking nerve fibers within the muscle sheath—closed manual "neurotripsy." Upon regeneration of the traumatized or broken nerve fibers, it is assumed that an increased number of axone branches may innervate a greater number of motor end plates. According to these authors, "neurotripsy," performed on muscles paralyzed by anterior poliomyelitis, has resulted in an increase in strength of approximately one half of a grade rated according to the Lovett System. Maximum effect was observed after about one year, although a considerable proportion of the total improvement was evident at 3 months. Muscles of low rating seemed to respond better than those of higher ratings. Increases in action potentials were parallel to increases in strength.

Prior to the report by these authors, Hines, Wehrmacher, and Thomson[2] had found that crushing the tibial nerve to the partially denervated gastrocnemius muscle of the rat resulted in a decrease in strength and weight of the muscle. Hodes[3] observed in patients with anterior poliomyelitis that there was a decrease in the action potentials of paretic muscles during the first four months following closed manual "neurotripsy." From 4 to 8 months after the operation the action potentials were slightly greater than those of controls and at more than 8 months there was an average increase in action potentials of 22%. Muscles which showed no electrical activity before the operation likewise showed no electrical activity after the operation.

This article is a preliminary report on studies which were undertaken to determine the effect of closed manual "neurotripsy" on the partially denervated anterior tibial muscle of the dog. The plan was to produce a partial, pure motor denervation of the anterior tibial bilaterally, to subject one anterior tibial to the operation, and to use the other as the control. For this experiment to be valid the following conditions would have to be fulfilled:

1. The strength and weight of the two normal anterior tibial muscles must be closely correlated.
2. The contribution of the ventral root of each spinal nerve supplying the anterior tibial must be determined.
3. The contribution of the ventral root of each spinal nerve must be the same for both left and right anterior tibial muscles.
4. Following bilateral section of identical ventral roots, the rate of atrophy and strength loss of the two muscles must be closely correlated.

*Method.* The partial denervation was accomplished by sectioning the ventral root of $L_6$ bilaterally. Closed manual "neurotripsy†" was performed on one anterior tibial muscle three months later and the animal sacrificed 5 months after the initial lesion. Autopsy was performed to check for any nerve regen-

* This work was aided by a grant from the National Foundation for Infantile Paralysis, Inc.

1 Billig, H. E., van Harreveld, A., and Wiersma, C. A. G., *J. Neuropath. and Exp. Neur.*, 1946, **5**, 1.

2 Hines, H. M., Wehrmacher, W. H., and Thomson, J. D., *Am. J. Physiol.*, 1945, **145**, 48.

3 Hodes, R., *Fed. Proc.*, 1948, **7**, 56.

† We wish to acknowledge the valuable instruction of Dr. R. T. McElvenny of the Department of Bone and Joint Surgery in the performance of this operative technic.

TABLE I.
Mean Weight and Total Strength in Grams of Anterior Tibial Muscles.

| | No. animals | Left | Right | t ratio | Correlation coef. |
|---|---|---|---|---|---|
| Total strength | 31 | 3870 | 4020 | 1.18 | .82 |
| Weight | 17 | 7.24 | 7.23 | .24 | .98 |

TABLE II.
Average Strength in Grams Developed by Anterior Tibial Muscles by Stimulation of Ventral Roots of Spinal Nerves.

| No. animals | Nerve stim. | Left | Right | t ratio | Correlation coef. |
|---|---|---|---|---|---|
| 21 | $L_4$ | 108 | 100 | .48 | .40 |
| 21 | $L_5$ | 504 | 509 | .05 | .83 |
| 20 | $L_6$ | 2810 | 3015 | .38 | .80 |
| 20 | $L_7$ | 342 | 323 | .20 | .23 |

TABLE III.
Average Strength and Weight in Grams of Anterior Tibial Muscles Following Bilateral Resection of Ventral Root $L_6$

| | 3 mos. after oper.; 6 animals | | | | 5 mos. after oper.; 5 animals | | | |
|---|---|---|---|---|---|---|---|---|
| | Left | Right | t ratio | Corr. coef. | Left | Right | t ratio | Corr. coef. |
| Strength direct stim. | 1917 | 2000 | 0.58 | .989 | 1800 | 1660 | .78 | .972 |
| Strength sciatic stim. | 1683 | 1883 | 1.45 | .990 | 1540 | 1400 | .55 | .948 |
| Weight | 5.56 | 5.23 | 1.41 | .979 | 5.14 | 4.92 | .49 | .915 |

eration and to verify the resected root. All animals showing any signs of regeneration were discarded. The contribution of the ventral roots supplying the anterior tibial muscle was determined in a group of normal dogs by recording the maximal tension developed by the muscle upon electrical stimulation of the individual ventral roots ($L_4$ to $L_7$ inclusive). Tension development was measured by a torsion myograph of the Blix type.

The control animals were subjected to a bilateral section of ventral root $L_6$, and with no further treatment they were sacrificed three and five months following the production of the original lesions. Wet weights and tension development upon maximal direct and indirect stimulation of the muscles were determined. Statistical analysis of the experimental data was carried out by the method of paired comparisons.

*Results.* Our experimental findings may be summarized as follows:

1. There was no significant difference between mean strengths and weights of the two normal anterior tibial muscles, and a high degree of correlation between the muscles of the same animal. (Table I).

2. $L_6$ supplied approximately 75% of the motor innervation to the anterior tibial. $L_4$, $L_5$, and $L_7$ supplied the remaining motor fibers (Table II).

3. In the individual animals the contribution of each $L_5$ and $L_6$ was practically the same for left and right muscles as indicated by the correlation coefficient (Table II).

4. At 3 months and at 5 months after bilateral resection of the ventral root of $L_6$ the loss of strength and degree of atrophy of the left and right anterior tibials was the same (Table III).

5. Two months after "neurotripsy," there was no significant difference in either weight or strength between the treated and untreated muscle (Table IV).

*Discussion.* Billig, van Harreveld, and Wiersma found that a considerable proportion of the improvement in their patients was evident at 3 months after "neurotripsy;" likewise, a better response was obtained on poorer muscles. The two-month interval following

TABLE IV.
Average Strength and Weight in Grams of Anterior Tibial Muscles After Neurotripsy on One Side.

| | 2 mos. after neurotripsy; 5 animals | | | |
|---|---|---|---|---|
| | Treated | Untreated | t ratio | Corr. coef. |
| Strength direct stim. | 1980 | 2140 | .68 | .819 |
| Strength sciatic stim. | 1700 | 1860 | .88 | .869 |
| Weight | 5.48 | 5.74 | .88 | .813 |

"neurotripsy" in the dog should be long enough to bring out any improvement resulting from the treatment. Our failure to demonstrate any such improvement in weight and strength of the treated muscles would indicate that in the dog this procedure does not increase the residual innervation of the partially denervated muscle. However, additional experimental animals with a greater degree of denervation are being run for a longer period of time following the "neurotripsy" procedure.

The results indicate that stimulation of the spinal nerves exclusive of $L_6$ yields approximately 1000 g of tension for each muscle. However, three months after bilateral resection of $L_6$, each muscle showed an average strength of approximately 1950 g and 5 months after bilateral resection a strength of approximately 1900 g. This may indicate that following partial denervation there may be an increase in the residual innervation as suggested by some investigators[2,4,5] or hypertrophy of the remaining innervated muscle fibers. This phenomenon is being investigated further.

*Summary.* Treatment of the partially denervated anterior tibial muscle of the dog with closed manual "neurotripsy" produces no significant increases in wet weight and muscle strength 2 months after treatment.

4 Weiss, P., and Edds, Jr., *Am. J. Physiol.*, 1946, **145**, 587.

5 Van Harreveld, A., *Am. J. Physiol.*, 1945, **144**, 477.

16803 P

## Isolation of Brucella from Apparently Healthy Individuals.

LEON V. MCVAY, FRANCES GUTHRIE, I. D. MICHELSON, AND DOUGLAS H. SPRUNT.
*From the Division of Pathology and Bacteriology, The University of Tennessee College of Medicine, Memphis, Tenn.*

In recent years the isolation of brucella organisms from cases of Hodgkin's disease[1] has caused considerable discussion. The confirmation of this finding in our laboratory and the statements in the literature that these organisms could be isolated from patients with various infections[2] led us to seek an explanation.

It seemed to us that the most likely explanation was that infection with brucella was widespread, at least in certain regions, and that the organism persisted in people furnishing favorable foci. In order to test this hypothesis it was decided to explore the possibility that tissues affording good conditions for growth of brucella organisms might actually harbor them. Since brucella are known

1 Parsons, P. B., and Poston, M. A., *South. Med. J.*, 1939, **32**, 7; Forbus, W. D., and Gunter, J. N., *South. Med. J.*, 1941, **34**, 376.

2 Mettier, S. R., and Kerr, W. J., *Arch. Int. Med.*, 1934, **54**, 702; Chaikin, N. W., and Schwinner, David, *Rev. Gastroenterol.*, 1943, **10**, 130; Amoss, H. L., *Internat. Clin.*, 1931, **4**, 93.

to multiply in macrophages and fibroblasts,[3] it was thought that cultures of enlarged prostates and fibrosed fallopian tubes might reveal the presence of these organisms.

At present 34 prostates have been cultured. From these cultures *Brucella abortus* has been isolated in 2 instances and *Brucella melitensis* in a third. Forty-three fallopian tubes have been similarly studied. From one of these *Brucella melitensis* has been isolated. The cultures of the prostates and tubes were made by obtaining from the surgeon, in a sterile container, portions of the prostate which were removed through the urethra. The specimens were immediately macerated, extracted, and the tissue extract incubated in Bacto-tryptose Broth at 37.5°C in 10% $CO_2$ for 10 days. After 10 days the broth specimens were inoculated on Bacto-Tryptose Agar plates, and these were similarly incubated for 10 days. Unless positive specimens were obtained sooner, this procedure was repeated twice before discarding the specimens as negative. Identification of the organisms was made by 1) the morphology of the colony on tryptose agar, 2) the morphology and staining reactions of the individual organisms, 3) sugar reactions, and 4) agglutination with specific antiserum. Two of the positive specimens were sent to another laboratory where their identification was confirmed.*

Microscopic examination of all the prostates and all the fallopian tubes was made. Practically all of them showed evidence of chronic infection. Sections from 2 of the prostates and the fallopian tube had a perivascular reaction which was not present in the others. This reaction consisted in a perivascular cuffing with mononuclear cells, many of which were eosinophilic. The third prostate could not be evaluated microscopically, because of extensive involvement with cancer.

Although none of the patients from whom brucella were obtained had any unusual symptoms prior to operation, a review of their histories revealed certain significant facts. In all 4 cases there was a history of country life and contact with cows and other farm animals. In 1 instance the patient had been in contact with an animal infected with brucellosis. In all cases there was a history of the consumption of raw milk. All of the patients had a clinical record of illnesses compatible with brucellosis. None of these illnesses, however, had occurred in recent months. The serum of all 4 patients lacked agglutinins for brucella, while their skin gave strongly positive reactions with brucella antigen. The post operative course of these patients was characterized by persistent low grade elevation of their temperature. It was impossible, however, in any of the cases to be certain that this fever was due to the brucella since in every instance there were other complications which might account for it.

We believe that this study, together with those of others, indicates that brucella is endemic, at least in certain parts of the world, and that the organisms may be carried by a number of apparently healthy individuals.

[3] Goodpasture, E. D., and Anderson, K., *Am. J. Path.*, 1937, **13**, 149; Castaneda, M. R., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 298.

* Two of the positive cultures were sent to the laboratory of Dr. W. W. Spink of the University of Minnesota School of Medicine where their identity was confirmed.

16804

# Effect of the Amino Acid Hexahomoserine on Growth and Hematopoiesis in Swine.

E. T. Mertz, W. M. Beeson, R. H. Waltz, Jr., and R. Gaudry.
(Introduced by W. A. Hiestand.)

*From the Departments of Agricultural Chemistry and Animal Husbandry,* Purdue University, Lafayette, Ind., and the Department of Biochemistry, Medical School, Laval University, Quebec, Canada.*

The synthesis of the amino acid, α-amino-ε-hydroxycaproic acid, was accomplished recently by one of the authors,[1] and the name hexahomoserine, was proposed for the new compound. It was shown that hexahomoserine could not replace lysine in the diet of rats, and that it was probably toxic.[2] Further studies[3] showed that the incorporation of hexahomoserine in the diet of the rat caused a drop in the hemoglobin level, red cell count, and red cell volume of the blood. Evidence was cited[3] indicating that hexahomoserine may be identical with the anemia-producing factor of deaminized casein.[4]

Inasmuch as feeding experiments with hexahomoserine have been limited to the rat, it appeared desirable to determine whether this compound would produce anemia in an unrelated species. The data presented below show that young pigs develop anemia and in addition, stop growing, when small amounts of hexahomoserine are added to an otherwise normal diet.

*Experimental.* Two male and 2 female purebred Duroc weanling pigs, whose weights ranged from 53-60 lb, were paired for the feeding experiments. The 4 pigs were kept in individual pens and were fed twice daily with amounts to satisfy the individual appetite of each animal. All animals received, throughout the course of the experiment, a ration consisting of ground corn 58.75%, ground wheat 20%, Purdue supplement V 20% (this supplement consists of meat and bone scraps 20%, fish meal 20%, soybean oil meal 40%, cottonseed meal 10%, and alfalfa leaf meal 10%), mineral mixture 1% (this consists of equal parts of limestone, steamed bone meal, and salt), and concentrated cod liver oil (NOPCO XX) 0.25%.

For a period of 27 days, male 72 and female 70 each received, in addition to the above diet, 3 g of DL-hexahomoserine daily. The hexahomoserine was mixed with the first half of the daily ration. The other pigs, male 73 and female 71, received only the basal ration.

A record was kept of daily food consumption and weekly weight gains. Twice a week red blood cell,[5] white blood cell,[5] platelet,† and reticulocyte[5] counts were made, and red blood cell diameters[5] were measured, in blood samples taken from a lancet wound in the ear tip. Once a week, red blood cell, hemoglobin,[6] cell volume,[7], icteric index,[5] prothrombin,[8] non-protein nitrogen,[9] total plasma pro-

* Journal paper No. 347, Purdue University Agricultural Experiment Station.

1 Gaudry, R., *Can. J. Research*, 1948, B, **26**, 387.

2 Gingras, R., Pagé, E., and Gaudry, R., *Science*, 1947, **105**, 621.

3 Pagé, E., Gaudry, R., and Gingras, R., *J. Biol. Chem.*, 1947, **171**, 831.

4 Hogan, A. G., Powell, E. L., and Guerrant, R. E., *J. Biol. Chem.*, 1945, **137**, 41.

5 Todd, J. C., and Sanford, A. H., *Clinical Diagnosis by Laboratory Methods*, pp. 238, 249, 294, and 386, W. B. Saunders Co., Philadelphia, 1936.

† Determined by measuring the ratio of platelets to red blood cells in blood diluted with 12% $MgSO_4$ and stained with Wright's stain.

6 Evelyn, K. A., *J. Biol. Chem.*, 1936, **115**, 63.

7 Wintrobe, M. M., *J. Lab. Clin. Med.*, 1929, **15**, 287.

8 Ziffren, S. E., Owen, C. A., Hoffman, G. R., and Smith, H. P., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 595.

9 Hawk, P. B., and Bergeim, O., *Practical Physiological Chemistry*, pp. 420 and 453, P. Blakiston Co., Philadelphia, 1937.

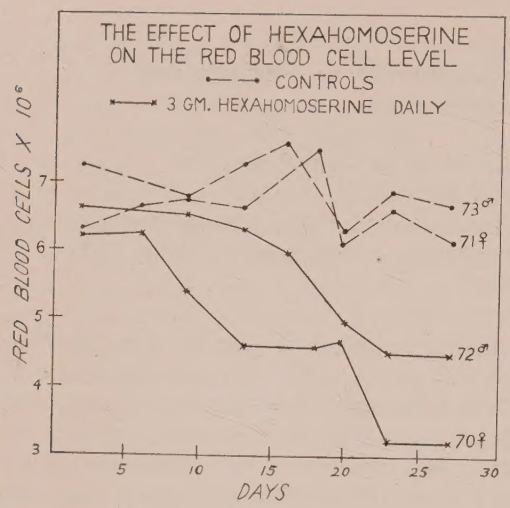



FIG. 1.

tein,[9] albumin,[9] globulin,[9] and fibrinogen[9] determinations were made on blood samples drawn from the anterior vena cava.

*Results.* The effect of hexahomoserine on the red blood cell level of the swine is shown in Fig. 1. The red blood cell level of pig 70 began to fall about 9 days after hexahomoserine had been added to the diet. By the 27th day, the red cell count had dropped to 3.2 million. At this point, the feeding of hexahomoserine was discontinued. One week after hexahomoserine was removed from the diet, the red cell count of this pig was still 3.2 million. The following day the pig died. Autopsy‡ showed marked enlargement of the spleen, lesions of swine pox, and a myocarditis characterized by accumulations of mononuclear cells. Microscopic examination of the bone marrow revealed no abnormal increase or reduction in the content of immature red blood cells. The bone marrow appeared to be functioning normally in the anemic animal.

The curve in Fig. 1 shows that pig 72 did not have a lowered erythrocyte count until about the 20th day after hexahomoserine had been added to the diet. On the 27th day, the last day on which the compound was fed, the erythrocyte count was 4.4 million, as compared with 6.6 million for the control pig 73. One week after the feeding of hexahomoserine had been discontinued, the erythrocyte count of pig 72 had risen to 5.1 million, and the hemoglobin level and cell volume were back to normal (15.3 g of Hb and 35.7 ml of total cells per 100 ml of blood compared with 15.2 g of Hb and 36.2 ml of total cells for pig 73). Ten days after the removal of hexahomoserine from the diet, the erythrocyte count of pig 72 had risen to 6.1 million. The animal was maintained on the basal ration for a total of 5 weeks after the hexahomoserine was dropped from the diet, and was then autopsied. Macroscopic and microscopic examination of the various tissues, including the bone marrow, showed no abnormalities; the ingestion of hexahomoserine had apparently caused no permanent damage.

The hemoglobin and cell volume values obtained on the blood of the four pigs are summarized in Table I. The blood of pig 70 shows a marked drop and the blood of pig 72 a slight drop in hemoglobin and cell volume.

The white blood cell, platelet and reticulocyte counts, the red blood cell diameter, and the icteric index, prothrombin, non-protein nitrogen, total plasma protein, albumin, globulin and fibrinogen values obtained on the blood of the two pigs receiving hexahomoserine showed no significant deviations from the values obtained simultaneously on the 2 normal control pigs. The experimental data are not reproduced here. It should be pointed out that the level of hexahomoserine fed in these studies may have been too low to produce changes in any blood constituent except the red blood cell.

In addition to depressing the level of red blood cells, hexahomoserine affected the growth rate of the pigs (Fig. 2). The compound appeared to have a delayed action on growth, for the two animals consumed normal quantities of feed and gained weight at a normal rate for approximately one week after the compound was added to the diet. After this initial period, however, both animals consumed less feed and stopped growing. During the last 18 days of the experiment, the animals

‡ We are indebted to Dr. L. P. Doyle, Department of Veterinary Science, Purdue University, for performing the autopsies on pigs 70 and 72.

TABLE I.
Effect of Hexahomoserine on Hemoglobin Level and Blood Cell Volume of Swine.

| No. of days on diet | Hemoglobin (g Hb/100 ml blood) | | | | Cell volume (ml cells/100 ml blood) | | | |
|---|---|---|---|---|---|---|---|---|
| | 70 ♀* | 71 ♀† | 72 ♂* | 73 ♂† | 70 ♀ | 71 ♀ | 72 ♂ | 73 ♂ |
| 0 | 12.8 | 15.1 | 14.9 | 12.4 | 31.0 | 36.5 | 36.0 | 31.0 |
| 6 | 14.4 | 14.3 | — | — | 29.5 | 33.0 | — | — |
| 12 | 9.9 | 14.8 | 13.2 | 15.2 | 29.7 | 35.9 | 32.8 | 39.4 |
| 18 | 10.3 | 15.0 | 12.0 | 15.6 | 25.0 | 34.6 | 29.6 | 35.6 |
| 27 | 6.4 | 15.0 | 12.5 | 14.7 | 22.5 | 39.5 | 32.0 | 39.0 |

* Animal received 3 g of hexahomoserine daily.
† Control animal. Received no hexahomoserine.

receiving hexahomoserine each consumed an average of 2.2 lb of feed per day, and gained little or no weight; the control animals each consumed an average of 3.5 lb of feed per day and gained weight in a normal manner.

Removing hexahomoserine from the diet of pigs 70 and 72 did not cause an immediate resumption of growth. Pig 70, when returned to the basal ration, lost 6 pounds during the first week and succumbed on the eighth day. Pig 72 recovered from the anemia in about 10 days but did not show normal weight gains for one month.

*Discussion.* The data presented above indicate that no major constituent of the blood except the red blood cell is affected when 3 g of DL-hexahomoserine are fed daily for 27 days to young (50-60 lb) pigs. If the decrease in circulating red cells in the blood of these pigs were due to a destruction of the red cells by hexahomoserine, one would expect an increase in the icteric index of the blood. No increase was observed. It is possible that hexahomoserine slows down or prevents the release of mature red cells to the blood stream. The finding of a normal supply of immature cells in the bone marrow of the anemic pig 70, and the presence of a normal icteric index and reticulocyte count in both of the anemic animals would support this hypothesis.

The inhibition of growth by hexahomoserine may be due to the development of a lysine deficiency in the animals. No data are available on the lysine requirements of swine, but lysine has been found essential for growth in all species tested to date. Hexahomoserine is closely related structurally to lysine, and may be a metabolic antagonist of the latter. Further studies on the mechanism of the action of hexahomoserine are in progress.*

*Summary.* Two Duroc pigs were fed hexahomoserine ($\alpha$-amino, $\epsilon$-hydroxy caproic acid) for a period of 27 days. A marked lowering of the red blood cell count was observed in both animals. No major constituent of the blood, except the red blood cell, was affected. In addition to developing anemia, the pigs made no significant weight gains after one week on the diet containing hexahomoserine, and it is concluded that the amino acid inhibits growth.

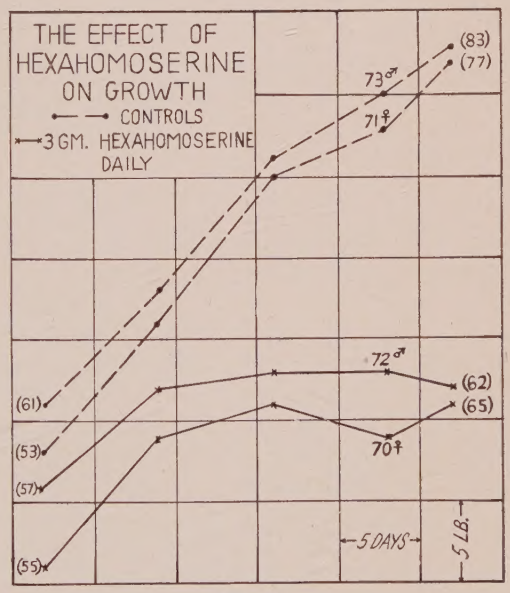



FIG. 2.
The numbers in parentheses denote the initial and final weights of the pigs.

* Since this paper was written, it has been shown conclusively that lysine is essential for the growth of pigs (Mertz, E. T., Shelton, D. C., and Beeson, W. M., *J. Animal Sci.*, 1948, **7**, 530).